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COLLEGE OF AGRICULTURE UNIVERSITY OF NEBRASKA
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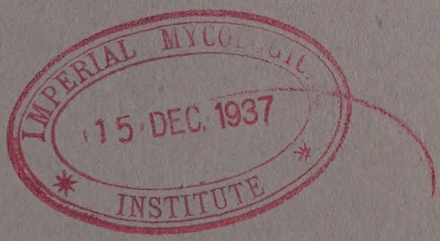
Comparative Physiology of Actinomyces in Relation to Potato Scab

Mitrofan M. Afanasiev
Department of Plant Pathology



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SUMMARY

1. A study of the physiology of parasitic and saprophytic Actinomyces was undertaken to obtain in general, a better understanding of their metabolism and if possible to find differences in their physiology which would be helpful in explaining the nature of their parasitism.

2. Twenty-five different cultures of Actinomyces were used in this investigation. Thirteen were isolated from scabby potatoes in Nebraska and seven of these were parasitic. The other twelve were all saprophytic and were obtained from different sources in this country and abroad.

3. Cultures A-1, A-12, *A. clavifer*, *A. setonii*, 3369 (*A. flavus*, A and B), 3372 (*A. viridis*), *A. tricolor*, and *A. xanthostroma*, which were reported by other investigators to be parasitic, failed to develop scab.

4. The potato scab which was produced by all seven parasitic cultures was of three different types: (1) common, (2) deep, and (3) russet. Two or three of these types were often present on individual potatoes. The difference in the scab exhibited by parasitic cultures was one of degree of pathogenicity rather than of type. These results do not agree with those of Millard and Burr (22), who reported that different cultures of Actinomyces produce different types of scab.

5. Studies proved that larvae of fickle midge (*Sciara inconstans* Fitch) by feeding on the dead tissues within the deep scab lesions make them appear larger, but that *A. scabies* is capable of producing deep-scab pits in the absence of these or other larvae.

6. Pathogenicity studies of culture N-23 on radishes, sugar beets, and sweet potatoes showed that it produced a slight infection on radishes but only a trace on sugar beets and none on sweet potatoes.

7. The growth and behavior of parasitic and saprophytic Actinomyces on different sources of carbon were about the same, except that all parasitic cultures were able to utilize sucrose and raffinose, while the majority of the saprophytes were unable to use these two sugars.

8. The growth and behavior of parasitic and saprophytic cultures of Actinomyces were similar on all media containing different nitrogenous compounds except on the medium with urea. Practically all parasitic and some saprophytic cultures failed to grow on a medium to which 0.5 per cent of urea was added. Detailed investigations showed that this failure was due to the toxicity of ammonia, which was produced as a result of decomposition of urea.

9. Further investigations showed that ammonium carbonate, ammonium bicarbonate, and ammonium hydroxide were also toxic to parasitic Actinomyces because of the evolution of ammonia from these compounds. These experiments established the fact that ammonia has a strong toxic effect on the growth of all parasitic and on some of the saprophytic Actinomyces.

10. The inhibition of growth as caused by potassium bicarbonate and calcium hydroxide on Actinomyces was also studied. These compounds had a greater inhibitory effect on parasitic than on saprophytic Actinomyces, and their direct toxicity inhibited growth of parasitic cultures more than did the high degree of alkalinity developed by these compounds.

11. The application of urea to soil inoculated with *A. scabies* inhibited the development of scab in proportion to the amount applied. No scab occurred when urea was used after sterilization and just previous to inoculation at the rate of 5 grams to 7 pounds of soil.

12. Studies of the effect of different carbon-to-nitrogen ratios showed them to have a pronounced effect on the amount of nutritive and aerial mycelium developed. There was very little difference in the development of nutritive mycelium of parasitic and saprophytic cultures except with a very small amount of carbon, in which case the parasitic cultures failed to grow. The differences in the development of aerial mycelium of parasitic and saprophytic cultures with different carbon-to-nitrogen ratios were pronounced. A high amount of nitrogen inhibited the production of aerial mycelium by the parasites but did not affect that of the saprophytes.

13. Studies of the ability of Actinomyces to produce soluble pigments on a tyrosine medium showed that all parasitic and some of the saprophytic cultures were able to produce melanin pigment on this medium but only in combination with other nitrogenous compounds and not when tyrosine was present alone. Different carbon compounds in the media had very little influence on the production of melanin pigment.

14. When media with amino acids other than tyrosine were used, Actinomyces failed to develop pigments comparable to melanin.

15. The ability of the parasitic cultures of Actinomyces studied in this investigation to utilize sucrose and raffinose, their inhibition by ammonia and other compounds, and their ability to produce melanin pigment in a tyrosine medium are facts of value in differentiating them from many saprophytic Actinomyces and contribute to our understanding of their physiology and control.

Comparative Physiology of Actinomyces in Relation to Potato Scab

MITROFAN M. AFANASIEV [1]

Practically every type of soil contains a large number of different Actinomyces, the majority of which are saprophytic, while others are parasitic on different crop plants such as potatoes, beets, and radishes. One of the most important of these diseases is potato scab, caused by *Actinomyces scabies* (Thaxter) Güssow.

Millard and Burr (22) have reported that there are a number of species of Actinomyces which are able to cause different types of potato scab. Lutman and coworkers (20) have concluded that pathogenicity is lost in the absence of the host and that in regaining its pathogenic habits the organism produces a milder scab, russeting. At the present time it is impossible to differentiate parasitic and saprophytic Actinomyces in any way other than by inoculation tests.

Lutman and Cunningham (19) in earlier work on potato scab concluded that all known strains of *A. scabies* are able to produce brown pigmentation on media containing proteins or potato juice. Drechsler (8) found that of the seventeen morphologically distinct saprophytic species which he studied, eleven exhibited a tyrosinase reaction. This last work indicates that not only parasitic but also saprophytic Actinomyces have the ability to produce the enzyme tyrosinase. Waksman (33) made an attempt to correlate the pathogenicity of different strains of *A. scabies* with their ability to produce melanin pigment on tyrosine medium, but his results were variable. Millard and Burr (22) indicate that some of the parasitic cultures which they used gave tyrosinase reaction and others did not. This is the only published work which indicates that some nonchromogenic Actinomyces are pathogenic on potatoes.

If a correlation between the ability of Actinomyces to produce melanin pigment and their pathogenicity could be established, it would be possible to determine pathogenicity without first testing them on host plants, and soil could be examined for its power to produce scab disease without growing a crop.

Sanford (28) obtained control of potato scab through the application of organic matter and believed that this occurred because of an inhibiting effect on *A. scabies* by the bacteria which developed in the green manure. Goss (11) demonstrated that in rotations under irrigation a crop which precedes potatoes has a direct influence on the amount of scab developed. Undoubtedly planting a succession of different crops in the same ground results in changes in the microflora of the soil, but the effect of these changes on the parasitic flora is not known. The answer to many phases of the potato scab problem is dependent upon a clearer understanding of

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the soil as a physico-chemical complex acting upon the microörganisms living in it, and upon a greater knowledge of the physiology of the soil microörganisms in general and their mutual relationships.

The resistance of different potato varieties to scab has been attributed to structural differences by Lutman and Cunningham (19) and Longreé (17), while Kiessling (13) and others consider resistance to be based on physiological factors. A better understanding of the nature of this resistance must be based upon a knowledge of the physiology of the scab organism itself and its relationship to the morphological and physiological peculiarities of different potato varieties.

The present work was undertaken with the purpose of investigating the physiology of different strains of *A. scabies* as compared with saprophytic Actinomyces. By a comparative study of these groups, it was thought that differences in the physiology of the two might be found which would give a better understanding of the nature of this parasitism and also of the metabolism of Actinomyces in general. In addition, it was hoped that the determination of the factors influencing the parasitism of Actinomyces might establish a basis for the development of more efficient methods of controlling scab diseases.

The present investigation is composed of the following phases: (1) a study of the pathogenicity of *A. scabies,* (2) the behavior of Actinomyces on different sources of carbon and nitrogen, (3) the effect of different carbon:nitrogen ratios on the development of Actinomyces, and (4) a study of the production of soluble pigments by Actinomyces on a tyrosine medium.

SOURCES OF CULTURES AND METHODS OF ISOLATION

Twenty-five different cultures of Actinomyces were used, twelve of which were isolated by the author from scabby potatoes in Nebraska and the remainder obtained from various sources in the United States and abroad.

Cultures received in 1931 from B. J. Dippenaar:

A-1.—This culture was isolated in 1928 from a scab lesion on a Flowerball potato, Stellenbosch, C. P., Union of South Africa. Dippenaar stated that this culture, when tested, produced an infection which was not severe.

A-12.—This culture was isolated in 1929 from a scab lesion on a Flowerball potato, George, C. P., Union of South Africa. Dippenaar stated that this culture produced a very severe infection.

Cultures received in 1930 from the Centraalbureau voor Schimmelcultures, Baarn, Holland:

A. clavifer W. A. Millard.—This culture was isolated by Millard (22) from a common, ruptured scab lesion on a Great Scott potato. In his test of pathogenicity, it produced an "intermediate" type of scab. The virulence of this culture was designated as "1".[2]

A. setonii W. A. Millard.—This culture was isolated by Millard (22) from an unruptured scab lesion on a British Queen potato. In his test of pathogenicity, it produced a "pitted" type of scab. The virulence of this organism was designated as "4."

[2] Millard and Burr used a scale of "1" to "12" for designating increasing degrees of pathogenicity of Actinomyces.

Cultures received in 1929 from the American Type Culture Collection, The John McCormick Institute for Infectious Diseases, Chicago, Illinois:

3372 (*A. viridis* Millard and Burr).—This culture was isolated by Millard and Burr (22) from a pitted, ruptured scab lesion on a Kerr's Pink potato. In their test of pathogenicity, it produced a pitted type of scab. The virulence of this culture was designated as "5."

3369 (*A. flavus* Millard and Burr).—This culture was isolated by Millard and Burr (22) from a large, slightly ruptured scab lesion on an unknown potato variety from Germany. In their test of pathogenicity, it produced a "tumulus" type of scab. The virulence of this culture was designated as "6."

This culture later was subdivided in Nebraska by R. W. Goss into the following two subcultures: 3369-A, having yellowish nutritive mycelium and whitish yellow to gray aerial mycelium, and 3369-B, having white nutritive mycelium and practically no aerial mycelium.

Cultures received in 1932 from F. M. Blodgett, Cornell University, who had originally obtained them from the Centraalbureau voor Schimmelcultures, Baarn, Holland:

A. flavus (Krainsky).—This culture is listed by Centraalbureau as one obtained from Waksman and Curtis. It is not known whether this culture was tested for pathogenicity.

A. tricolor Wollenweber.—This culture was originally isolated by Wollenweber (36) from a "Flachschorf" on a Magnum Bonum potato and when tested by him for pathogenicity, it produced the same type of scab.

A. xanthostroma Wollenweber.—This culture was isolated by Wollenweber (36) from the two types of scab, "Buckelschorf" and "Flachschorf," on an Up to Date potato. When tested by him for pathogenicity, it produced the same two types of scab.

The following three cultures were obtained from different sources:

3374 (*A. praecox* Millard and Burr).—Received in 1935 from S. Waksman, New Jersey Experiment Station. This culture was isolated by Millard and Burr (22) from a large, unruptured "knob-like" type of scab lesion on an Allay potato. Millard and Burr found it to be nonpathogenic.

N-23.—This culture was isolated by R. W. Goss, University of Nebraska, in 1929 from a deep scab lesion on a Bliss Triumph potato. It has always produced a severe infection of the common and deep types of scab with the latter type predominating.

1356 (*A. scabies* (Thaxter) Güssow).—Received in 1930 from the National Collection of Type Cultures, the Lister Institute, England. This culture was isolated and identified by Dr. E. J. Butler, Imperial Mycological Institute, Kent, Surrey, England. The characters of this culture were quite different from those of all other strains of *A. scabies* studied. It is probable that the culture had become mixed and that hyphal tip isolations eliminated the pathogenic species from the culture.

The following cultures were isolated by the author in 1931 and 1932, from potatoes grown in western Nebraska: cultures 79, 86, 87, 103, 104, and 106 from deep scab lesions and 105 and 107 from common scab lesions on Bliss Triumph potatoes; cultures 88-A, 88-B, 89, and 90 from severe type of russet scab on Irish Cobbler.

METHOD OF ISOLATION

The method of isolating Actinomyces described by Shapovalov (29) was employed in this investigation. Colonies of Actinomyces usually appeared in three to four days and after microscopic examination a number of colonies were transferred to potato dextrose agar in test tubes. When a good growth had developed, the cultures were carefully examined and the most prevalent types were selected for stock cultures.

METHOD OF ISOLATING SINGLE HYPHAL TIPS

In the literature on Actinomyces, there is no indication that the investigated cultures were started from single spores. Because of the difficulty of differentiating one Actinomyces from another, and because of their minuteness, it was considered that single-spore isolations or their equivalents were necessary before physiological studies could be started.

Because of the minuteness of the spores and the absence of a micromanipulator, it was necessary to make isolations by cutting off the tips of their aerial hyphae. The method employed in making these hyphal-tip isolations was a modification of methods described by Brown (6) and Edgerton (9). It is being described elsewhere by the author and was employed for all cultures studied in the present investigation with the exception of culture 3374 (*A. praecox*), which was received after all the other isolations had been made.

PATHOGENICITY TESTS

POTATOES

Pathogenicity tests were conducted with three objectives in mind: (1) To compare the relative virulence of cultures isolated from scabby potatoes grown in Nebraska with others obtained from investigators in the United States and abroad, (2) To test the pathogenicity of unknown cultures of Actinomyces isolated from potato tubers infected with different types of scab, (3) To test the relationship of different cultures of Actinomyces to the type of scab they produce as stated by Millard and Burr (22).

Methods.—The following method was used for the pathogenicity tests. Healthy Bliss Triumph potatoes were treated with hot formaldehyde 1:120, at 51°-52° C. for three to four minutes and left over night under burlap sacks soaked in the same solution. When dry they were cut into seed pieces weighing approximately 45 grams each and left in a damp atmosphere to suberize. The seed pieces were sprouted in sterile sand and transplanted to sterilized soil (3 parts of compost soil, 1 part of manure, and 1 part of sand) in 7-inch clay pots in saucers. When the planting was made in the fall or early winter, the seed pieces were treated with a 2.5 per cent ethylene chlorhydrine solution to break their dormancy. The soil, pots, and saucers were steamed for one to two hours and then subjected to 20 to 25 pounds of steam pressure for approximately three hours in the autoclave.

Fifteen potato plants were used for testing the pathogenicity of each culture of Actinomyces. At the time of planting, the soil was inoculated with a suspension of spores and mycelium, obtained by scraping 25 two-weeks-old petri-dish cultures of Actinomyces grown on two per cent potato dextrose agar. The inoculum used per pot was 150 cc. of suspension, or the equivalent of 1.7 petri-dish culture per pot. All plants were grown in an isolated room in a greenhouse having a cement floor and cement tables, neither of which were ever swept with a broom, as this had been

found in preliminary experiments to result in infection of noninoculated controls. Instead, the floor and tables were regularly washed with water containing a strong disinfectant. Sterile tap water was used for watering the plants in the pots. These series of 15 plants each were grown during the winters of 1933-34, 1934-35, and 1935-36. The temperature of the greenhouse was maintained as nearly as possible at 22° C., except during the latter part of the season when it became considerably higher than the desired average because of high outside temperature.

When the plants were mature the tubers were dug, washed, and divided into the grades listed in Table 1 according to the severity of infection. Scab was recorded on the basis of both weight and number of tubers, but as the results were similar, only one method is reported in the tables.

The different types of scab were designated as follows:

1. **Russet.**—The periderm of the tuber was slightly darkened and roughened. The scab lesions were distributed either singly over the potato surface or in masses which covered large areas on the tuber surface. The margins of these areas were not well defined and the type of scab lesion was not distinct. It is not yet known how much importance can be attributed to the scab organism in the production of this type of scab, and how much to other fungi or to certain environmental conditions. For this reason, consideration was given chiefly to common and deep types of scab.

2. **Common.**—The periderm of the potato tuber was broken by circular, oval, or irregular scab lesions with their surfaces at the level or slightly above that of a healthy periderm.

3. **Deep.**—The periderm was broken by circular, oval, or irregular scab lesions, whose depths varied from slight depressions to pulpy pits 4 to 5 mm. deep.

Results.—Of the twenty-five Actinomyces cultures whose pathogenicity was tested on potato tubers, the following seven were unquestionably parasitic: N-23, 79, 86, 87, 104, 105, and 107 (Table 1). These seven cultures which were isolated from scabby potatoes in Nebraska, produced common and deep types of scab, with the latter type predominating. Practically all of the deep-scab lesions contained whitish mycelium. Russet scab was present to a slight extent on the majority of potato tubers harvested from sets inoculated with the above-mentioned seven cultures.

The following seven cultures of Actinomyces, 89, 90, 103, 106, 3369-A (*A. flavus* Millard and Burr), *A. flavus* (Krainsky), and *A. setonii* were considered to be questionable parasites because none, except culture 106, produced a significant amount of scab. The slight infection of deep scab which was produced by culture 106, occurred on only seven potatoes out of 42 and these were all from one plant. It is possible that this infection was caused by contamination.

The eleven remaining cultures, A-1, A-12, 88-A, 88-B, 1356 (*A. scabies?*), 3369-B (*A. flavus*), 3372 (*A. viridis*), 3374 (*A. praecox*), *A. clavifer, A. tricolor,* and *A. xanthostroma,* were nonpathogenic. Common and deep types of scab were absent, but russet scab occurred to a very slight degree

TABLE 1.—*The pathogenicity of different cultures of Actinomyces on potato tubers of the Bliss Triumph variety.*[1]

Extent of scab	Parasitic cultures							Questionable parasitic cultures						
	N-23	79	86	87	104	105	107	89	90	103	106	3369-A *A. flavus*	*A. flavus* (Kr.)	*A. setonii*
Tubers														
Number	83	46	75	45	38	76	91	35	48	56	42	30	55	31
Grams	2750	1270	1720	1410	1320	2090	2330	690	1610	1510	1230	910	1560	940
Percentage of surface area infected [2]														
0	0.0	5.5	0.0	48.2	30.3	0.0	0.0	95.7	98.1	92.9	81.3	93.4	96.1	89.4
0– 2	3.7	34.7	6.4	44.7	22.7	2.4	0.0	4.3	1.9	7.1	12.2	6.6	3.9	10.6
2– 25	31.0	37.0	24.0	3.6	22.7	16.7	9.0	0.0	0.0	0.0	2.5	0.0	0.0	0.0
25– 75	38.0	15.8	54.6	3.5	16.7	64.6	35.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0
75–100	27.3	7.0	14.6	0.0	7.6	16.3	55.8	0.0	0.0	0.0	4.0	0.0	0.0	0.0
Percentage of scab classified as:														
Common (*p. ct.*)	25	25	50	25	50	50	25	100	100	100	0	100	100	0
Deep (*p. ct.*)	75	75	50	75	50	50	75	0	0	0	100	0	0	100
Russet scab [2]	S	VS	S	VS	0.0	VS	S	VS	VS	VS	VS	VS	VS	VS

Extent of scab	Saprophytic cultures										
	A-1	A-12	88-A	88-B	1856 *A. scabies* (?)	3369 B *A. flavus*	3372 *A. viridis*	3374 *A. praecox*	*A. clavifer*	*A. tricolor*	*A. xantho-stroma*
Tubers											
Number	35	29	53	27	33	25	37	50	41	32	30
Grams	1400	1150	1580	1280	1040	360	930	1210	620	1020	770
Percentage of surface area infected [2]											
0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
0– 2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
2– 25	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
25– 75	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
75–100	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Percentage of scab classified as:											
Common (*p. ct.*)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Deep (*p. ct.*)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Russet scab [2]	0.0	0.0	S	S	0.0	0.0	0.0	0.0	0.0	0.0	0.0

[1] Fifteen soil pots were used for testing the pathogenicity of each culture of Actinomyces. The noninoculated sets of potatoes grown in the same room with the above ones remained healthy.

[2] Scab data were also recorded on the basis of the number of tubers, with results similar to these on the weight basis.

[3] Abbreviations: S slight, VS very slight.

with 88-A and 88-B. On the basis of their ability to produce russet scab, these two cultures could be placed in the group of questionable parasites.

The two groups, the saprophytes and questionable parasites, were composed of cultures obtained from various sources and from isolations made by the author. (For convenience, all questionable parasites will be called saprophytes in all further discussions and in all tables except Table 1.) Some of them, which had been reported by other authors to be parasitic, failed to produce scab to any appreciable degree.

Culture 1356 (*A. scabies?*) which was isolated and identified by Butler in England, did not behave like strains of *A. scabies* isolated in Nebraska either in the test of pathogenicity or in culture. It is not at all unlikely that the culture became contaminated before it was received. It is not known who isolated culture *A. flavus* (Krainsky), or whether its pathogenicity had previously been tested. Both of Wollenweber's cultures, *A. tricolor* and *A. xanthostroma,* and both of Dippenaar's cultures, A-1 and A-12, which were reported to be parasitic, failed to develop any scab in the author's test.

Millard and Burr reported cultures *A. clavifer, A. setonii,* 3369 (*A. flavus,* 3369-A and 3369-B) and 3372 (*A. viridis*) to be parasitic and 3374 (*A. praecox*) to be saprophytic. In the author's investigation, *A. clavifer,* 3369-B (*A. flavus*), 3372 (*A. viridis*) and 3374 (*A. praecox*) did not produce scab at all and *A. setonii* and 3369-A (*A. flavus*), developed such insignificant amounts that they cannot be regarded as parasitic. Possibly the small amount of infection which did occur was caused by accidental contamination.

It is very difficult to explain the results obtained with Wollenweber's, Dippenaar's, and Millard and Burr's cultures, which were reported to be pathogenic, as none of these developed scab in the author's tests under conditions which resulted in a high percentage of infection with other strains of *A. scabies.* It is possible that after being cultivated for long periods of time on artificial media they had lost their virulence, but practically all of the author's cultures retained their original virulence after they had been cultivated on artificial media for five to eight years.

The author does not know the potato varieties used by Dippenaar and by Millard and Burr for their tests. Possibly they used more susceptible varieties than the Bliss Triumph, and even though their cultures were weak parasites they were able to develop considerable amounts of scab.

In their publication, Millard and Burr (22) report that "Stock subcultures were made at regular intervals of one month. After varying lengths of time some of these cultures showed a lack of vitality which evinced itself in a falling off of spore production, or in loss of pigment formation. When this occurred, the culture was replated and new cultures made. Not all the colonies obtained by this practice show a complete return of vigour. Thus, it was found that a culture producing a deep stain on protein media may, when plated out, give certain colonies which stain as deeply as the original culture and others which do not stain at all.

A number of new cultures must be prepared and a selection made. All our stock cultures were replated in this way from time to time."

In the present investigation, the chromogenic cultures isolated by the author retained their ability to produce pigments at all times. The ability of different cultures to sporulate did not seem to vary greatly, especially when grown on potato dextrose agar which was used in propagating stock cultures.

It is interesting to note that other strains of *A. scabies* obtained from workers in this country and tested in Nebraska, but which were not used in this investigation, appeared to be similar to all seven parasitic cultures isolated in Nebraska and were all parasitic on potatoes. Another strain of *A. scabies* obtained from Dippenaar, which originally came from Millard through the Centraalbureau in Holland, was proved to be pathogenic in tests not shown in Table 1.

Since one of the objectives of this investigation was to test the relationship of different cultures of Actinomyces to the types of scab they produce, as reported by Millard and Burr, an attempt was made to obtain all their cultures, but these were not available. Millard and Burr state that *A. setonii* and 3372 (*A. viridis*) developed "pitted" types of scab lesions and 3369 (*A. flavus*) "tumulus" types of scab lesions. In the present investigation there was no evidence that the different cultures used produced different types of scab lesions. All types of scab were produced by each parasitic culture, and usually different types of scab could be found on individual potatoes. The deep type of scab predominated. The only difference that occurred was one of degree of pathogenicity. It is probable that the host reaction has as much to do with the type of scab as have the different organisms. If this is true, one could hardly expect an exact similarity in every pustule produced by an individual organism; rather, there would be a gradation of types, with the deep type resulting from the most virulent cultures.

From disease readings of potato scab taken in Nebraska over a period of several years, it was found that in a very dry year such as 1934 when tubers grew slowly, practically no deep scab was present. In other years deep scab occurred on the same potato varieties, grown in the same soil, probably because of the effect of environmental conditions through its influence on the host.

In some of the preliminary tests of pathogenicity, fickle midge larvae (*Sciara inconstans* Fitch) were often found in deep-scab lesions and it appeared that they might play an important role in the development of this type of scab lesion. Do they live only on tissues infected with the scab organism or are they capable of independently causing lesions on tubers? To answer these questions the following modifications of the test of pathogenicity were made, using only cultures 79, 87, and *A. flavus* (Kr.).

Fifteen pots of soil for each of these cultures were prepared as previously described. Four were covered with a fine grade of muslin and placed in water containers to prevent possible infestation of the soil by

fickle midge larvae. The remaining eleven served as controls. The potato tubers from the four covered pots were harvested separately.

In both the covered and uncovered pots culture 79 developed severe deep scab, culture 87 medium scab with the deep-type scab predominating, and *A. flavus* (Kr.) developed practically no scab at all. No larvae occurred in the uncovered pots inoculated with culture 87 and *A. flavus* (Kr.). In the uncovered pots with culture 79, larvae were present and the scab lesions were slightly larger than in the covered pots where larvae were absent. The interiors of these lesions were smooth and the soil particles within the pit could be easily removed. In contrast to this, the deep-scab lesions in which larvae were absent were slightly smaller and filled with soil that adhered closely to the infected tissues.

It is evident, then, that deep-scab lesions can be produced by the action of the scab organism alone without the assistance of fickle midge larvae. Undoubtedly these larvae live on infected tissues and probably slightly enlarge the cavity by feeding on the dead potato tissues.

PATHOGENICITY OF CULTURE N-23 ON RADISH, SUGAR BEET, AND SWEET POTATO

So far, parasitism on potatoes alone, and not other plants, has been considered. In order to establish this point, the author inoculated radishes, sugar beets, and sweet potatoes. Culture N-23 was used for the inoculations and also the same type of soil. Pots or crocks were used as in the previous experiments with potatoes, and everything was sterilized as before. When the plants were mature they were dug and graded for scab infection according to the following grades: *healthy, very slight* (with only small, undeveloped scab lesions), *slight* (with one to two pronounced scab lesions on each radish), *medium* (with more than two and not more than five lesions on each radish), *severe* (with more than five lesions on each radish).

Radish (*Raphanus sativus* L.).—Ten seven-inch pots were planted with seeds of the White Icicle variety of radish and ten others with the Early Scarlet Globe variety. Seven pots of each variety were inoculated as soon as the seeds germinated and the plants emerged. The inoculum was prepared as in the potato experiments. The remaining three pots of each variety were left as checks.

From this experiment (Table 2), it was evident that culture N-23 was pathogenic on radish. The scab which developed was rather slight. The White Icicle variety seemed more susceptible than Early Scarlet Globe.

Sugar beet (*Beta vulgaris* L.).—Sugar beet seeds were planted in 20 crocks, each of 10-liter capacity. When the beet seeds began to germinate, the soil in 15 of the crocks was inoculated with culture N-23 at the rate of two petri-dish cultures per crock. The five remaining crocks of beets were left as checks.

When harvested only traces of infection, in the form of undeveloped lesions, were found on five out of the 14 inoculated beets (Table 3). The checks were all healthy.

The results of this experiment show that either culture N-23 was only slightly pathogenic on beets, or the conditions which resulted in infection on potatoes were different from those required for beets.

TABLE 2.—*The pathogenicity of A. scabies N-23 on radishes* (*Raphanus sativus* L.).

Variety	Treatment	No.	Wt.	Severity of infection [1]				
				Healthy	Very sl.	Slight	Medium	Severe
		No.	*Grams*	*P. ct.*	*P. ct.*	*P. ct.*	*P. ct.*	*P. ct.*
White Icicle	Inoculated	55	1345	65 0	17 8	14 9	2 3	0 0
White Icicle	Control	36	810	100.0	0.0	0.0	0.0	0.0
Farly Scarlet Globe	Inoculated	65	1300	93.2	4.6	0.7	1.5	0.0
Early Scarlet Globe	Control	36	650	100.0	0.0	0.0	0.0	0.0

[1] Percentages based on weights.

TABLE 3.—*The pathogenicity of A. scabies N-23 on sugar beets* (*Beta vulgaris* L.).

Variety	Treatment	No.	Wt.	Severity of infection [1]				
				Healthy	Very sl.	Slight	Medium	Severe
		No.	*Grams*	*P. ct.*	*P. ct.*	*P.ct.*	*P. ct.*	*P. ct.*
Sugar beets	Inoculated	14	2960	62.7	37.3	0.0	0.0	0.0
Sugar beets	Control	5	1050	100.0	0.0	0.0	0.0	0.0

[1] Percentages based on weights.

Sweet potato (*Ipomoea batatas* Poir.).—Fifteen sweet-potato plants were grown and inoculated in a manner similar to that of previous tests but no infection occurred. Evidently culture N-23 was either nonparasitic on sweet potatoes, or the conditions of growth were not favorable for infection.

In these tests the failure to obtain infection on sweet potatoes or more than very slight infection on beets may have been due either to unfavorable conditions in the soil or to the fact that scab on these plants may be caused by other species or strains of Actinomyces.

ABILITY OF ACTINOMYCES TO UTILIZE DIFFERENT CARBON COMPOUNDS

This study was undertaken mainly to determine if differences exist between parasitic and saprophytic Actinomyces in their ability to utilize various sources of carbon and nitrogen. The results will be compared with those of Waksman (32 33), who also reviewed the work done by earlier investigators on these subjects. His work dealt with Actinomyces in general, without any particular consideration of their pathogenicity.

Twenty-five different parasitic and saprophytic Actinomyces (Table 1) were tested with different sources of carbon. Through such a study it was thought that certain facts might be established which could be correlated with the ability of *A. scabies* to parasitize potato tubers. R. W. Goss in histological studies of potato tissues infected with scab (unpublished),

found hyphae of parasitic Actinomyces within the cells. The organism may penetrate the cell walls by fermenting the substances of which they are mainly composed, *i.e.* suberin, lignin, cellulose, and pectin.

The ability of Actinomyces to utilize other sources of carbon such as dextrose, maltose, sucrose, raffinose, starch, araban, xylan, and agar was also studied. The majority were tested in an agar medium, so that it was necessary to investigate the ability of Actinomyces to utilize agar itself as a source of carbon.

The reaction of all media, except the one containing pectin, was adjusted before sterilization to pH 7.0. Twenty-four different cultures of Actinomyces were tested on media containing pectin, cellulose, lignin, and suberin, and each was grown for two months. Twenty-five cultures were used for testing the remaining substances and were grown in duplicate test tubes for a period of only one month. During this period several readings of the amount of nutritive and aerial[3] mycelium, color of aerial mycelium, and the production of soluble pigments were made. In this investigation the amount of growth and intensity of pigmentation were designated by the following numerals: 0—none, 1—very slight, 2—slight, 3—fair, 4—good, and 5—very good. The final readings are given in the tabulated results.

Pectin.—Considerable work has been done on the ability of soft-rot-producing bacteria to ferment pectin. Jones (12) determined that *Bacillus carotovorus* destroyed the middle lamella of soft vegetables and Smith (30) reported this same ability for *Bacterium campestre*. More recently, Wolf (35) studied the ability of soft-rot-producing bacteria and others to utilize pectin in culture media. He judged the ability of the different organisms to ferment pectin substances by the formation of pectic acid in the medium.

In this study, the method used was based largely on that of Wolf (35). One per cent of purified pectin was added to two different kinds of media. The first medium, A, was of the following composition:

Peptone	2.0 g.	$MgSO_4 \cdot 7H_2O$	0.5 g.	$FeSO_4$	0.01 g.	Agar	20.0 g.
KH_2PO_4	1.0 g.	KCl	0.5 g.	Pectin	10.0 g.	Dist. water	1,000 cc.

The second medium, B, was of the same composition as used by Wolf:

Peptone	10.0 g.	NaCl	5.0 g.	Agar	20.0 g.
Liebig's beef ext.	3.0 g.	Pectin	10.0 g.	Dist. water	1,000 cc.

Both media were sterilized in the autoclave and cooled to approximately 60° C. before sterile pectin was added. The reaction of medium A was adjusted to pH 7.0 and that of medium B to pH 7.7 by the colorimetric method. Both were distributed to test tubes in 10 cc. amounts and to each was added 0.5 cc. of phenol red indicator (0.02 per cent). Media A and B were also used without the indicator as checks to see if the indicator had any effect on the growth of Actinomyces. One test tube each of media A and B of both sets (with and without indicator), was used for each one of the tested Actinomyces cultures. The ability of Actinomyces to utilize

[3] The terms "nutritive" and "aerial" as applied to mycelium are used in the same sense as by Drechsler (8).

TABLE 4A.—*Growth of Actinomyces,*[1] *pectin medium A, with phenol red indicator*[2]*—comparative growth of mycelium, pigmentation of medium, and pH of medium at various intervals.*

Intervals	Parasitic cultures							Saprophytic cultures																
	N 23	79	86	87	104	105	107	89	90	103	106	3369-A *A. flavus*	*A. flavus* (Kr.)	*A. eidonii*	A-1	A-12	88-A	88-B	1356 *A. scabies* (?)	3369-B *A. flavus*	3372 *A. viridis*	*A. claviger*	*A. tricolor*	*A. xantho-stroma*
Two days																								
Mycelium:																								
Nutritive	3	2	2	2	3	4	3	3	3	4	4	4	4	4	3	3	2	3	4	2	3	2	3	3
Aerial	1	6	0	1	1	3	1	1	1	3	3	1	0	4	0	2	0	1	3	0	1	0	1	1
pH in medium:																								
Upper	7 5	7 4	7 1	7 4	7 3	6 7	7 0	6 7	7 5	7 2	6 6	6 8	7 2	7 4	7 4	7 3	7 0	-6 6	7 7	7 2	7 2	7 2	6 6	7 4
Lower	7 5	7 4	7 3	7 4	7 4	7 3	7 4	7 3	7 5	7 3	7 2	7 2	7 4	7 4	7 5	7 3	7.4	7 3	7.5	7 4	7 4	7 3	7 3	7.4
Eight days																								
Mycelium:																								
Nutritive	4	4	3	3	4	5	4	3	4	5	4	5	4	5	4	4	4	4	5	3	5	3	4	5
Aerial	2	1	2	2	2	4	1	2	3	4	3	3	0	5	3	4	4	4	4	0	4	1	3	4
pH in medium:																								
Upper	7 6	7 8	7 6	7 6	7 9	8 0	7 8	6 6	7 6	8 0	8 0	8 0	8 0	7 9	7 6	7 8	8 0	8 0	8 0	7 6	8 0	7 6	7.4	8 0
Lower	7 3	7 3	7 3	7 3	7 3	7 2	7 4	7 3	7 5	7 3	7 1	7 2	7 4	7 3	7 3	7 1	7 3	7.2	7 5	7 2	7.4	7.2	7 2	7 4
Eleven days																								
pH in medium:																								
Upper	7 8	8 2	7 8	8 2	8 2	8 4	8 2	7 6	8 2	8 4	8 4	8 4	8 4	8 4	8 2	8 2	8 2	8 4	8 4	8 2	8 4	8 2	8 2	8 4
Lower	7 3	7 3	7 3	7 3	7 3	7 3	7 3	7 3	7 3	7 5	7 3	7.3	7 5	7 5	7 3	7 3	7 3	7.3	7.5	7.3	7.5	7 3	7 3	7 5
Forty-three days																								
pH in medium:																								
Upper	8 4	8 4+	8 4	8 4	8.4	8 4	8.4-	8 4+	8 4+	8 4+	8.4+	8.4+	8 4+	8 4+	8 4+	8 4+	8 4+	8 4+	8 0	8 4+	8 4+	8 4+	8 4	8 4+
Lower	7 4	7 6	7 4	7 4	7 4	7 4	7 9	7 6	8 1	8 1	7 9	8 4+	8 1	8 1	7 6	7 9	8 1	8 4+	8 0	7 6	7 9	8 1	7 6	8 1
Fifty-seven days																								
Nutritive	4	5	5	4	4	5	4	5	5	5	4	4	3	5	5	5	5	5	5	3	4	3	5	5
Aerial	3	4	4	4	3	5	3	5	5	5	4	3	2	5	5	5	5	5	5	0	5	4	5	5
Mycelium:																								
pH in medium: [3]																								
Lower	7 6	8 4-	7 6	7 6	8 4	8 4	8 4+	7 6	8 4+	8 4+	8 2	8 4+	8.2	8.4	8 4+	8.4	8.4+	8 4+	8 2	8 4	8 4+	8 2	8 2	8 4+
Color of aerial mycelium [4]	WG	WG	WG	WG	WG	WG	WG	W	WG	CW	C	YG	G	C	WG	C	G	G	C	0	DG	G	BIG	W

[1] The amount of growth is designated by the following numerals: 0—none, 1—very slight, 2—slight, 3—fair, 4—good, and 5—very good.
[2] Check tubes without phenol red showed the same amount of growth.
[3] The pH was above 8.4 in the upper portion of every culture.
[4] Color symbols are: W—white, G gray, C—cream, Y yellow, Bl—blue, D—dark.

TABLE 4B.—*Growth of Actinomyces,*[1] *pectin medium B, with phenol red indicator*[2]*—comparative growth of mycelium, pigmentation of medium, and pH of medium at various intervals.*

Intervals	Parasitic cultures							Saprophytic cultures																
	N-23	79	86	87	104	105	107	89	90	103	106	3369-A *A. flavus*	*A. flavus* (Kr.)	*A. setonii*	A-1	A-12	88-A	88-B	1356 *A. scabies* (?)	3369-B *A. flavus*	3372 *A. viridis*	*A. clavifer*	*A. tricolor*	*A. xantho-stroma*
Two days																								
Mycelium:																								
Nutritive ...	3	4	4	3	3	4	4	3	5	5	4	4	4	5	4	4	3	4	4	4	4	2	4	4
Aerial	1	0	0	0	0	0	0	0	2	4	4	3	0	4	0	3	0	0	3	0	1	0	2	1
Pigmentation ..	4	3	4	3	3	3	2	0	3	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0
pH in medium:																								
Upper	7.4	7.4	7.2	7.4	7.5	7.4	7.5	6.9	7.6	7.8	7.8	7.8	7.8	7.8	7.5	7.8	7.1	6.7	7.8	7.8	7.7	7.5	6.7	7.7
Lower	7.4	7.4	7.4	7.4	7.5	7.4	7.5	7.4	7.5	7.5	7.4	7.5	7.5	7.3	7.5	7.2	7.4	7.4	7.6	7.5	7.4	7.4	7.4	7.3
Eight days																								
Mycelium:																								
Nutritive ...	4	4	4	4	3	4	4	5	5	5	5	4	5	5	5	4	4	4	5	4	4	4	4	4
Aerial	2	1	0	0	2	3	0	5	4	5	4	3	0	5	1	5	2	1	4	0	3	0	4	3
Pigmentation ..	4	3	4	3	3	3	3	0	3	0	0	0	0	0	0	0	4	2	0	0	0	0	0	0
pH in medium:																								
Upper	8.2	8.2	8.2	8.2	8.4	8.2	8.2	8.2	8.2	8.4	8.4	8.4	8.4	8.4	8.4	8.4	8.4	8.4	8.4	8.4	8.4	8.4	8.4	8.4
Lower	7.7	7.5	7.5	7.4	7.2	7.4	7.6	7.5	7.5	7.7	7.7	7.6	7.6	7.2	7.7	7.1	7.2	7.4	7.8	7.3	7.7	7.6	7.3	6.8
Eleven days																								
pH in medium: [3]																								
Lower	7.7	7.6	7.7	7.7	7.6	7.6	7.7	7.3	7.7	7.9	7.9	7.7	7.7	7.9	7.7	7.3	7.6	7.7	7.9	7.3	7.9	7.7	7.7	7.3
Fifty-seven days																								
Mycelium:																								
Nutritive ...	4	4	5	4	4	4	4	5	5	5	5	4	5	5	5	5	4	3	5	4	4	4	5	4
Aerial	3	3	0	0	2	3	0	5	5	5	4	5	0	5	2	5	3	2	5	0	5	0	5	5
Pigmentation ..	4	3	4	3	4	4	4	0	3	0	0	0	0	0	0	0	4	2	0	0	0	0	0	0
Color of aeriel mycelium [4] ...	WG	WG	0	0	WG	WG	0	G	WG	C	C	YG	0	C	WG	C	G	G	W	0	DG	0	BIG	W

[1] The amount of growth and intensity of pigmentation are designated by the following numerals: 0—none, 1—very slight, 2 slight, 3—fair, 4—good, and 5—very good. When pigmentation occurred in the medium it was always dark.

[2] Check tubes without phenol red showed the same amount of growth.

[3] The pH was above 8.4 in the upper portion of every culture and after 43 days it was above this point throughout the culture medium in every case.

[4] Color symbols are: W white, G—gray, C—cream, Y—yellow, Bl blue, and D dark.

pectin was judged by the change in the reaction of the medium. Results of this experiment are presented in Tables 4A and 4B.

Medium A, with a pH of 7.6 at the start, showed a reaction close to the neutral point after growth began, and in some cases was even slightly acid. Two days after inoculation a slightly lower pH was recorded in the upper part of the medium in the test tube than in the lower part, due to the fact that growth started in the upper part and fermentation of pectin into pectic acid occurred there first.

Six days after these conditions were noted, the upper part of the medium showed an increase in alkalinity, while the lower part showed a slight decrease. This can be explained by the fact that in the upper part of the medium where growth was developing, the organisms, while fermenting pectin into pectic acid, used it, so that there was no free pectic acid present while some free pectic acid accumulated in the lower part of the medium. Later readings showed that both parts had become more alkaline, with the upper part increasing its alkalinity more rapidly than the lower. The increase in alkalinity in the lower part no doubt was due to the fact that the organisms had started to utilize pectic acid from this part of the medium. The final reaction of the upper part of the media of all cultures exceeded pH 8.4, which is the upper limit for phenol red. The reaction of the lower part of the media of some was as high as the upper part, while for others it was lower than pH 8.4. This increase in alkalinity undoubtedly was due to the metabolic activity of Actinomyces in this medium.

No pigment was produced by any of the Actinomyces cultures in this medium. The color of aerial mycelium is presented in Tables 4A and 4B. There were no significant variations in the color of aerial mycelium throughout these experiments, so no additional description of this will be made.

Growth and behavior of the cultures on medium B were similar to those on medium A, except that the alkalinity of medium B increased more rapidly, probably because it had a much larger supply of available nitrogen, and for this reason, in a corresponding period of time, more pectic acid was used. In addition to this, all parasitic and three saprophytic cultures developed a dark discoloration in this medium. Growth on the media without indicator was practically identical to that stated above.

It is concluded from this study that the Actinomyces tested were able to use pectin as a source of carbon. No particular differences were displayed by parasitic and saprophytic Actinomyces.

Cellulose.— The parasitism of many organisms has been associated with their cellulose-dissolving power. Waksman (32), in his study of the ability of Actionomyces to utilize cellulose, used four different methods. The results of these four different tests, as he pointed out, were not identical, but without question he confirmed the results of previous authors on the ability of Actinomyces to utilize cellulose. No data were found in the literature as to whether any differences exist between parasitic and saprophytic Actinomyces in their ability to use cellulose.

Two different methods were used to test the utilization of cellulose by Actinomyces. In the first, a suspension of cellulose was prepared as described by McBeth and Scales (21). One per cent of this cellulose was added to a basic medium of the following composition:

KH_2PO_4	1 g.	KCl	0.5 g.	KNO_3	3.0 g.	Agar	20.0 g.
$MgSO_4 \cdot 7H_2O$	0.5 g.	$FeSO_4$	0.01 g.	Cellulose	10.0 g.	Dist. water	1,000 cc.

In the second, filter paper was suspended in a solution of the same basic medium. This was varied by adding 0.1 per cent of dextrose, in order to start cellulose fermentation and growth, if a more available source of carbon was needed. One test tube each of the three types of media was used for each of the Actinomyces tested. The tabulated results of this experiment are presented in Table 5 and can be summarized as follows:

Cellulose agar.—With few exceptions, growth on this agar was fair and in some instances, very good. All parasitic cultures and some saprophytic cultures produced a slight discoloration in this medium. There were no significant differences in the behavior of parasitic and saprophytic Actinomyces.

Liquid media with and without dextrose.—Two variations of this method were used.

Solution with 0.1 per cent of dextrose.—Growth was fair on the filter paper of this medium and appeared to be even more abundant on the liquid itself. All Actinomyces developed slight pigmentation in this solution.

Solution without dextrose.—Growth on this medium was similar to that on the previous solution but in general it was less extensive. The parasitic cultures developed slightly more growth than the saprophytic. A number of cultures developed a slight pigmentation in this solution.

Lignin.—Lignin from two sources was tested. Lignin "A" was prepared from corn cobs by an alkaline method,[4] and lignin "B" was made by the author from corn cobs by the method described by Phillips (24). Lignins "A" and "B" were added separately to the same basic agar medium as was used for cellulose fermentation, at the rate of 5.0 grams per liter. The medium was sterilized in flowing steam because it was found that lignin decomposed if sterilized under pressure. One test tube each of both types of media was inoculated with each one of the cultures. The results of this experiment are presented in Table 5. A similar amount of growth, which was fair, was developed by the majority of the cultures on both of these media. No pigmentation occurred on either media and no differences were noted between parasitic and saprophytic cultures.

From this experiment it is evident that Actinomyces were able to use lignin as a source of carbon, although not as easily as the simpler carbon compounds.

Suberin.—Suberin was tested because of the protective role it plays in potato tubers. Common cork, which is practically pure suberin, was cut into small thin strips and a piece was placed at the bottom of each test

[4] The author is indebted to Dr. M. Phillips of the United States Department of Agriculture for the lignin used in this medium.

TABLE 5.—*Growth of Actinomyces on media containing cellulose, lignin, and suberin.*[1]

Media	Parasitic cultures							Saprophytic cultures																
	N-23	79	86	87	104	105	107	89	90	103	106	3369-A *A. flavus*	*A. flavus* (Kr.)	*A. setonii*	A-1	A-12	88-A	88-B	1356 *A. scabies*(?)	3369-B *A. flavus*	3372 *A. viridis*	*A. clavifer*	*A. tricolor*	*A. xantho-stroma*
Cellulose agar																								
Mycelium:																								
Nutritive ...	3	4	3	3	5	3	3	3	5	4	4	3	2	5	4	4	3	3	4	3	3	1	4	2
Aerial	2	3	3	3	4	3	3	3	4	4	4	3	0	4	3	3	3	2	4	0	3	0	3	2
Pigmentation...	2D	2D	2D	1D	2D	2D	2D	0	2D	0	0	0	0	0	0	0	1D	0	0	0	0	0	2Bl	0
Cellulose solution (filter paper + 0.1 per cent dextrose)																								
Mycelium on paper:																								
Nutritive ...	2	3	5	3	5	2	5	3	4	4	2	3	2	3	2	3	4	4	2	1	3	2	3	1
Aerial	3	4	5	4	5	2	4	3	5	5	3	0	0	3	3	3	4	4	2	0	2	1	3	2
Mycelium in liquid:																								
Nutritive	5	5	5	5	5	5	5	5	3	5	5	5	4	5	5	4	5	5	4	5	4	4	5	4
Aerial	4	4	5	4	5	4	5	2	4	5	5	3	2	4	3	3	5	5	3	0	4	1	5	2
Pigmentation ...	1Y	1Y	1Y	1Y	1Y	2Y	1Y	1Y	1Y	1Y	2Y	3Y	1Y	1Y	1Y	2Ca	1Y	1Y	3Y	1Y	2Ca	1Y	3Bl	1Y
Cellulose solution (filter paper without dextrose)																								
Mycelium on paper:																								
Nutritive ...	2	2	2	4	5	1	5	2	4	2	1	1	1	2	3	1	1	1	1	0	1	1	2	1
Aerial	3	3	1	4	4	1	5	3	5	4	1	1	0	4	4	1	1	1	1	0	0	0	3	1
Mycelium in liquid:																								
Nutritive ...	4	4	3	4	5	4	5	5	3	4	3	3	3	4	4	4	3	3	2	2	3	2	5	2
Aerial	4	4	3	3	5	3	5	3	4	3	2	1	0	4	3	3	2	2	2	0	2	0	4	0
Pigmentation...	1Y	1Y	1Y	0	0	1Y	1Y	0	2Y	0	1Y	0	0	0	0	2Y	0	0	1Y	0	0	0	2Bl	0
Lignin agar A[2]																								
Mycelium																								
Nutritive	4	3	3	3	3	4	2	1	3	5	4	2	3	5	3	3	3	3	4	1	3	1	5	3
Aerial	3	2	3	2	2	4	1	0	3	4	3	0	0	4	2	2	2	3	4	0	1	0	4	4
Lignin agar B[2]																								
Mycelium																								
Nutritive	3	2	3	2	2	4	2	2	4	4	5	3	3	5	2	3	4	3	4	1	5	1	5	3
Aerial	1	1	2	0	2	3	1	2	3	3	4	3	1	5	3	4	3	2	4	0	4	0	5	3
Suberin solution																								
With 0.1 per cent dextrose																								
Mycelium on cork:																								
Nutritive .	4	0	3	4	0	5	4	3	0	3	0	2	3	4	2	2	3	3	0	3	3	3	3	4
Aerial	4	0	2	4	0	4	5	4	0	4	0	1	3	5	4	1	4	4	0	0	3	1	2	4
Mycelium in liquid:																								
Nutritive .	5	0	1	3	0	4	4	3	0	2	3	4	3	5	1	2	4	3	3	2	3	2	2	3
Aerial	4	0	0	3	0	4	2	1	0	2	0	2	1	5	0	0	3	1	0	0	2	0	1	3
Without dextrose																								
Mycelium on cork:																								
Nutritive .	3	3	0	3	3	4	4	3	2	4	3	1	2	4	2	3	0	5	2	0	4	4	4	3
Aerial	3	4	0	1	3	3	4	4	2	3	3	0	3	3	2	3	0	5	1	0	4	0	4	3
Mycelium in liquid:																								
Nutritive .	2	3	0	4	3	4	4	4	2	4	3	4	4	4	4	4	0	5	4	0	3	3	4	4
Aerial	1	3	0	0	2	2	3	3	1	1	2	0	0	3	5	3	0	4	3	0	2	0	3	3

[1] The amount of growth and intensity of pigmentation are designated by the following numerals: 0—none, 1—very slight, 2—slight, 3—fair, 4—good, and 5—very good. Color of pigmentation is indicated by letters: Bl—blue, Y—yellow, Ca—carmine, G—green, and D—dark.

[2] See page 19 for source of lignin.

tube to which the basic medium was added, so that about a half centimeter of the cork appeared above the surface of the solution.

In one series of cultures, 0.1 per cent of dextrose was added to show whether it would have a stimulating action on the initiation of growth on the cork, and in the other series the medium was used without dextrose. The results of this experiment are presented in Table 5.

In general there appeared to be very little difference in the growth of the different cultures on these media containing cork. The majority of the organisms developed as much growth on the cork as on the solution and some of the organisms which failed to grow in one medium developed a fair amount of growth in the other. No pigmentation was developed in these media and there were no noticeable differences between the parasitic and saprophytic cultures in their growth on cork.

In testing the remaining sources of carbon, namely 1 per cent dextrose, 1 per cent maltose, 1.5 per cent sucrose, 1 per cent raffinose, 1 per cent araban, 1 per cent xylan, 1 per cent starch, and 2 per cent agar, the same basic medium employed as for testing cellulose, lignin, and suberin was used. Agar media with araban, xylan, and sucrose were sterilized in the autoclave. The rest of the carbon-containing substances, including 2 per cent of sucrose added to the liquid basic medium, were sterilized for three successive days in dry heat at 105° C. and then added to a previously sterilized basic medium. The reaction of the media was adjusted to pH 7.0 before sterilization. The results of this experiment are presented in Table 6.

Dextrose.—Three cultures, 88-A, 88-B, and *A. tricolor,* out of the twenty-four tested, failed to grow on this medium but the rest developed good growth. Parasitic cultures in general developed slightly less aerial mycelium that the saprophytic cultures. Culture A-12 developed a slight pink pigmentation.

Maltose.—Except for a few cultures, all developed good growth on this medium, the only difference being that the parasitic cultures developed slightly less aerial mycelium than the saprophytic cultures. Three cultures developed pigmentation in this medium.

Sucrose.—In comparing the amount of growth produced by the saprophytic cultures on sucrose agar and in sucrose solution (Table 6), it is evident that some of the cultures which produced no growth at all in a sucrose solution, developed a fair amount of growth on sucrose agar. This can be explained by the fact that the sucrose agar was sterilized in the autoclave after the sucrose was added, and it is possible that certain amounts of it were hydrolyzed into simpler sugars which the saprophytes used for their growth and for this reason the discussion will be based upon the results obtained with the liquid medium.

All the parasitic cultures produced good growth but only 5 of the 18 saprophytic cultures showed growth in the liquid medium. These cultures, 90, *A. flavus* (Kr.), A-1, 88-A, and 88-B, developed about the same amount of growth as the parasitic cultures.

TABLE 6.—*Growth of Actinomyces on media containing different sources of carbon.*[1]

Sources of Carbon	Parasitic cultures							Saprophytic cultures																
	N-23	79	86	87	104	105	107	89	90	103	106	3360 A *A. flavus*	*A. flavus* (Kc.)	*A. scabiei*	A-1	A-12	88-A	88-B	1356 *A. scabiei* (?)	3360 B *A. flavus*	337 *A. viridis*	337-1 *A. griseus*	*A. claviger*	*A. violaceus*	*A. xanthosporus*
Dextrose																									
Mycelium:																									
Nutritive	4	4	4	4	5	5	5	5	4	5	5	5	5	5	5	5	0	0	3–2	4	5	5	4	0	3–2
Aerial [2]	3–2	2-1	0	1	4	4	2–1	5	1	5	5	5	3	5	3–2	5	0	0	3–1	0	5	5	5	0	3–2
Pigmentation	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2P	0	0	0	0	0	0	0	0	0
Maltose																									
Mycelium:																									
Nutritive	4	5	4	4	5	4	5	5	4	5	5	4	5	5	5	3	5	5	2	5	5	5	4	5	2
Aerial	3–2	3	4	2-1	5	5	2	4	4	5	5	5	3	5	5	3	5	3	1	0	5	5	5	5	1
Pigmentation	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4PY	0	0	0	0	3G	0	0	5Bl	Pp 0
Sucrose in agar [3]																									
Mycelium:																									
Nutritive	5	5	5	2	5	5	5	3	5	1	4	4	4	1	5	1	1–0	1–0	1	0	2–1	1	4	1	0
Aerial	3	4	2–1	1	4	5	3	3	4	1–0	3	4	4	1	4–3	1	0	0	1	0	1	0	4	1	0
Pigmentation	1B	1B	3B	0	1B	1B	1B	0	1B	0	1Y	0	0	0	0	0	0	0	0	0	0	0	0	1P	0
Sucrose in solution [3]																									
Mycelium:																									
Nutritive	4	2	3	3–2	3–2	2	4	0	1	0	0	0	3	0	3	0	2–1	3	0	0	0	0	0	0	0
Aerial	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Fehling's test	5	4	5	4	5	5	5	0	5	0	0	0	3	0	5	0	5	5	0	0	0	0	0	0	0
Raffinose																									
Mycelium:																									
Nutritive	5	5	4	4	5	5	5	0	3	1	0	0	5	0	5	0	4	3	0	0	0	0	0	3	0
Aerial	4–3	3	4–3	1	5	5	5	0	3	0	0	0	2	0	5	0	4	3	0	0	0	0	0	3	0
Pigmentation	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3Bl	0

[1] The amount of growth and intensity of pigmentation was designated by the following numerals: 0—none, 1—very slight, 2—slight, 3—fair, 4—good, and 5—very good. Color of pigmentation is indicated by the letters: P—pink, Y—yellow, G—green, Bl—blue, Pp—purple, C—cream, and B—brown.

[2] Two numerals are used to show the variation in growth of cultures in the duplicate test tubes.

[3] The results on the sucrose agar were probably due to hydrolysis of the sucrose during sterilization. In the sucrose solution the sugar was added after sterilization (see page 21). None of the cultures produced pigmentation in sucrose solution.

TABLE 6.—(*Concluded*).

Sources of Carbon	Parasitic cultures							Saprophytic cultures																
	4473	29	86	87	104	105	107	89	90	103	106	3868-1 *A. bovis*	*A. bovis* (N.)	*A. israelii*	A-1	A-12	88-A	88-B	1876 *A. israelii* (?)	3868-2 *A. bovis*	3872 *A. israelii*	3874 *A. israelii*	*A. thalifer*	[illegible]	[illegible]
Araban																								
Mycelium:																								
Nutritive	4	4	3	3	4	3	5	2	3	4	3	3	3	4	4	4	5	4	3	2	4	3	2	4	1
Aerial	3	3	3	3	4	1	4	2	3	4	3	2	2	3	4	3	4	3	3	0	4	2	0	2	0
Pigmentation	1B	1B	2B	1B	1B	1B	1B	0	1B	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Xylan																								
Mycelium:																								
Nutritive	4	3	3	3	4	3	3	4	4	4	5	3	3	4	3	4	4	4	4	3	5	4	2	3	5
Aerial	2	2	1	2	4	3	2	2	4	4	5	3	0	5	4	5	3	2	4	0	5	3	1	2	4
Pigmentation	1B	1B	1B	1B	1B	1B	1B	0	1B	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Starch																								
Mycelium:																								
Nutritive	5	5	5–4	4	5	5	5	5	3	5	5	4	5	5	5	5	5	3	5	5	5	4	5	[illegible]	[illegible]
Aerial	1	2	1	1	4	4	2	5	2	5	5	4	4	5	3	5	4	4–3	2	0	5	4	4	5	1
Pigmentation	0	0	1B	0	0	0	0	0	0	0	1C	0	0	0	0	1P	0	0	0	0	0	0	0	4B1	0
Diastat. activ. (Lugol)	2	2	3	3	2	4	3	4	2	4	5	4	3	5	2	5	4	5	4	3	4	5	2	3	4
Agar																								
Mycelium:																								
Nutritive	0	0	0	0	0	1	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
Aerial	0	0	0	0	0	1	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0

At the end of the experiment, a qualitative determination was made, by means of Fehling's solution, of the amount of reducing sugars present. This determination established the fact that in all cases where growth occurred, reducing sugars were present in large amounts, while they were absent when growth failed to occur. Checks tested at the beginning and at the end of the experiment showed no reducing sugars. The reaction of the medium at the end of the experiment deviated very little from the original neutral point. But with a few exceptions this experiment emphasized the fact that there was a pronounced difference between the saprophytic and the parasitic cultures of Actinomyces in their ability to use sucrose.

TABLE 7.—*Quantitative study of utilization of sucrose by parasitic and saprophytic cultures of Actinomyces.*

Time	Parasitic cultures			Saprophytic cultures			Check
	N-23	86	107	A-12	3374 *A. praecox*	*A. setonii*	
Total mgs. sugars in 10 cc.:							
At beginning	196	196	196	196	196	196	196
At end	188	181	184	193	196	196	196
Total mgs. reducing sugars in 10 cc.:							
At beginning	1.4	1.4	1.4	1.4	1.4	1.4	1.4
At end	2.9	2.6	4.3	1.0	1.1	0 9	1.5
Growth [1]	S to M	S to M	M	None	None	None	None

[1] Abbreviations: S—slight, M—medium.

A detailed quantitative study of the ability of Actinomyces to use sucrose was undertaken, in which the three saprophytic cultures, A-12, 3374 (*A. praecox*), *A. setonii* and the three parasitic cultures, N-23, 86, and 107, were used. The same liquid medium with two per cent of sucrose was used, and the medium, including sucrose, was sterilized for three successive days in the steamer. The reaction was adjusted to pH 7.0. Two 500 cc. Erlenmeyer's flasks containing 200 cc. of this medium were used for each one of the organisms and they were each inoculated with 1 cc. of a water suspension of spores and mycelium. Two flasks with the same amount of the solution were left as checks. The duration of the experiment was about six weeks. At the beginning and end of the experiment, determinations of total sugars and reducing sugars in the solution were made. The presence of reducing sugars was determined by using thiosulphate, and total sugars by the manometric method as described by Blish, Sandstedt, and Astleford (3) and improved by Sandstedt and Blish (27). For each 10 cc. of the solution to be tested for total sugars, 500 mgs. of yeast were used. Usually the maximum pressure in the manometer was obtained after five to six hours of incubation. For more details of the method and apparatus used, see Sandstedt and Blish (27).

The results are presented in Table 7. The parasitic cultures developed slight to medium growth, while none of the saprophytic cultures developed any growth. At the beginning of the experiment, the amount of total sugars was equivalent to 1.96 per cent, or 196 mgs. for each 10 cc. of

the solution, and at the same time, 0.014 per cent or 1.4 mgs. of reducing sugars were found for each 10 cc. of the solution.

The presence of reducing sugars might be explained by the fact that the sucrose which was used carried traces of reducing sugars, or because of prolonged steaming a small amount of sucrose was hydrolyzed.

The amount of total sugars, as shown by tests made at the end of the experiment, remained unchanged in the checks and in two saprophytic cultures. The third culture, A-12, showed a very slight decrease in amount of total sugars. The parasitic cultures used 8 to 15 mgs. of total sugars per 10 cc. of solution, or 160 to 300 mgs. per 200 cc. of the medium, which originally had 3,920 mgs. of total sugars.

The amount of reducing sugars in the checks remained practically unchanged, but the saprophytic cultures showed a slight decrease, probably because the organisms used it to a slight extent but not sufficiently to start visible growth. Parasitic cultures showed from two to three times more reducing sugars at the end of the experiment than at the beginning.

In general, it is evident that while the parasitic cultures were able to use sucrose, the saprophytes were unable to utilize this source of carbon. How general this may be for all Actinomyces is not known.

Waksman, in his studies on the metabolism of Actinomyces (32), stated that "only a few of the organisms were found to be able to invert the sucrose of the medium" and in another instance he mentioned that "even the organisms not producing invertase, seem to use it to some extent." He made no reference to the pathogenicity of the organisms.

Raffinose.—The results obtained with this medium were comparable to the results for sucrose. All parasitic cultures developed good growth, while the majority of the saprophytic cultures failed to grow. This similarity in behavior on sucrose and raffinose is quite significant, because these sugars are much alike. They are nonreducing sugars and the same enzyme, invertase, converts them into simple compounds which can be easily used by both types of Actinomyces. Only one culture, *A. murior*, developed pigmentation (blue) in this medium.

Araban and xylan.—Growths on these media were approximately alike, and while a few cultures showed a maximum, the majority had only a fair or slight growth. All parasitic cultures and saprophytic culture 90 produced a slight dark-brown discoloratoin in both of these media. Aside from the above, there were no differences between the parasitic and saprophytic cultures.

Starch.—Except for a few cultures, practically all developed a maximum of nutritive mycelium on this medium. The saprophytic cultures developed aerial mycelium more abundantly than the parasitic cultures. A few cultures produced pigmentation in this medium.

In addition to growing these cultures on medium in test tubes, they were grown on the same kind of medium, in duplicate petri dishes, for each culture. One dish was used for testing diastatic action with Lugol's solution, and the other, to which brom cresol purple

had been added, was used to test the production of acid. The results are presented in Table 6.

The growth of Actinomyces in petri dishes was about the same as in the test tubes. The diastatic action varied greatly and it is possible to say that, in general, the parasitic Actinomyces showed weaker diastatic action than did the saprophytic. No acid was produced as a result of starch fermentation.

Agar.—When agar alone was used as a source of carbon, only three cultures out of the whole group produced any growth. Culture 90 developed a slight growth and cultures 105 and *A. tricolor* only traces. Probably this was due to the fact that the inoculum for all of these test tubes, which was taken from cultures grown on potato dextrose agar, had a slight amount of this agar transferred to the test tubes with the inoculum, thereby giving these cultures material for growth.

It is possible to conclude that in all these experiments in which Actinomyces were grown on different sources of carbon, agar itself did not interfere with the testing of these organisms, because, as shown above, they were unable to use agar as a source of carbon.

Conclusions.—The compounds used in this study can be listed in the following order, according to the ability of Actinomyces to utilize them: starch, maltose, pectin, dextrose, xylan, araban, lignin, suberin, cellulose, raffinose, sucrose, and agar.

In general, the results of this investigation and those of Waksman follow the same lines. Actinomyces grew better on a solid medium than in solutions and the only difference between the parasitic and the saprophytic was in their behavior toward sucrose and raffinose. All parasitic cultures were able to grow on these media, while the majority of the saprophytic cultures could not utilize these sources of carbon. How much importance this characteristic has in explaining the pathogenicity of Actinomyces is not known. The ability of parasitic organisms to use sucrose, while saprophytic organisms can not, was observed previously by Clara (7) for a group of green-fluorescent bacteria.

ABILITY OF ACTINOMYCES TO UTILIZE DIFFERENT NITROGENOUS COMPOUNDS

Eleven different nitrogenous compounds were added to equal portions of the same basic medium (see page 19) which was used in testing the ability of Actinomyces to ferment cellulose and other carbon compounds. The compounds and amounts used were as follows: 0.3 per cent potassium nitrate, 0.3 per cent sodium nitrate, 0.3 per cent ammonium sulphate, 0.5 per cent asparagine, 0.1 per cent phenylalanine, 0.1 per cent histidine-hydrochloride, 0.1 per cent tryptophane, 0.1 per cent tyrosine, 0.5 per cent blood albumin, 0.5 per cent globulin, and 0.5 per cent urea. Dextrose was used (1.5 per cent) as a source of carbon. Before sterilization, the reactions of the eleven media were adjusted to pH 7.0 by the colorimetric method. Twenty-five different parasitic and sapro-

phytic cultures were grown for one month on each of the eleven media and several readings of the amount of nutritive and aerial mycelium, the color of the aerial mycelium, and the presence of soluble pigments in the media, were made. Because there was no significant variation in the color of the aerial mycelium produced here from that produced on carbon compounds, further discussion of this matter will not be made. Readings taken on the last date are presented in Table 8 and the summarized results are as follows:

Potassium nitrate.—All cultures grew fairly well on this medium except the saprophytic cultures 88-A, 88-B, and *A. tricolor,* which produced only traces of growth. The other saprophytic cultures developed slightly more aerial mycelium than the parastic cultures. A-12, 105, and 3372 (*A. viridis*) developed soluble pigment.

Sodium nitrate.—Growth of the cultures on this medium was similar to that on the medium containing potassium nitrate.

Ammonium sulphate.—In general, all cultures produced much less growth on this medium than on the two previous ones and the development of aerial mycelium was particularly poor. The parasitic cultures developed a very slight amount of aerial mycelium, while only a few of the saprophytic cultures produced any. The amount of growth in general was only slight to fair, probably because of the unfavorable shift in reaction toward the acid side resulting from the decomposition of ammonium sulphate and the consequent formation of sulphuric acid. No soluble pigments were produced.

Asparagine.—Practically all cultures grew fairly well on this medium. All the parasitic and a number of the saprophytic cultures developed soluble pigments of different colors. In general, the saprophytic cultures developed slightly more aerial mycelium than did the parasitic cultures.

Phenylalanine.—The majority of the cultures grew fairly well on this medium except the saprophytic cultures 88-A, 88-B, and 89, which displayed very poor growth. Only *A. flavus* (Kr.) developed pigmentation, and this to only a very slight degree. There were no significant differences between the two groups of organisms.

Histidine-hydrochloride.—Practically all cultures grew comparatively well on this medium. A number of the saprophytic cultures developed a slight pigmentation of various colors. No differences in growth were displayed by the two groups of organisms.

Tryptophane.—The growth of practically all cultures on this medium could be considered as only fair. Aerial mycelium was produced by the majority of the parasitic cultures but only by a few of the saprophytes. Only *A. tricolor* developed a slight pigmentation in this medium.

Tyrosine.—Practically all cultures grew fairly well on this medium and there were no significant differences between the two groups of organisms. Culture A-12 developed a slight yellowish pigmentation.

TABLE 8.—*Growth of Actinomyces on different sources of nitrogen.*[1]

Sources of Nitrogen	Parasitic cultures							Saprophytic cultures																	
	N-23	79	86	87	104	105	107	89	90	103	106	3369-A *A. flavus*	*A. flavus* (Kr.)	*A. setonii*	A-1	A-12	88-A	88-B	1356 *A. scabies*(?)	3369-B *A. flavus*	3372 *A. viridis*	3374 *A. praecox*	*A. claviger*	*A. tricolor*	*A. xantho-stroma*
Potassium nitrate, 0.3 per cent																									
Mycelium:																									
Nutritive	5	4	4	5	5	5	4	5	4	5	5	4	5	5	5	5	1	1	2	3	5	..	4	1	2
Aerial	2	3	2	3	4	4	2	5	4	5	5	5	4	5	5	5	0	0	4	0	5	..	5	0	4
Pigmentation	0	0	0	0	0	1C	0	0	0	0	0	0	0	0	0	1C	0	0	0	0	4G	..	0	0	0
Sodium nitrate, 0.3 per cent																									
Mycelium:																									
Nutritive	4	5	4	5	5	5	5	5	5	5	5	5	5	5	5	5	1	1	2	3	5	..	4	2	3
Aerial	2	3	2	4	5	4	1	5	4	5	5	5	3	5	5	5	0	0	1	0	5	..	5	0	1
Pigmentation	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3Pp	0	0	0	0	4G	..	0	0	0
Ammonium sulphate, 0.3 per cent																									
Mycelium:																									
Nutritive	3	2	3	4	3	4	3	4	3	3	2	2	3	4	3	4	3	2	2	3	2	..	2	3	4
Aerial	1	1	1	2	0	2	1	0	2	2	2	0	0	3	0	0	0	0	0	0	0	..	0	0	1
Pigmentation	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	..	0	0	0
Asparagine, 0.5 per cent																									
Mycelium:																									
Nutritive	5	4	4	5	4	5	5	5	5	5	5	4	4	5	5	5	4	4	5	4	5	5	5	5	5
Aerial	3	2	1	4	3	5	4	2	3	5	5	4	0	5	5	5	2	1	4	0	5	4	5	4	5
Pigmentation	1D	3D	3D	1D	1D	2B	1D	0	1D	2P	0	0	0	0	0	1P	2D	0	2YP	0	2G	0	0	3Bl	0
Phenylalanine, 0.1 per cent																									
Mycelium:																									
Nutritive	4	3	4	3	4	4	4	1	3	4	4	3	4	4	4	2	1	1	4	3	4	2	2	3	4
Aerial	4	1	4	3	3	4	3	0	3	3	3	1	2	0	4	0	0	0	2	0	5	0	0	3	4
Pigmentation	0	0	0	0	0	0	0	0	0	0	0	0	1Y	0	0	0	0	0	0	0	0	0	0	0	0
Histidine-hydrochloride, 0.1 per cent																									
Mycelium:																									
Nutritive	4	3	3	4	4	5	4	2	3	4	4	4	5	5	4	4	4	4	4	2	5	3	4	3	4
Aerial	4	3	4	3	4	5	3	0	3	3	3	2	3	5	5	5	2	3	4	0	5	1	3	3	4
Pigmentation	0	0	0	0	0	0	0	0	0	0	0	1Y	1Y	0	0	1C	0	0	1Y	0	1G	0	0	1Bl	0

[1] The amount of growth and intensity of pigmentation are designated by the following numerals: 0—none, 1—very slight, 2—slight, 3—fair, 4—good, and 5—very good. Pigmentation of the medium is indicated by letters: C—cream, G—green, Pp—purple, D—dark, B—brown, P—pink, Y—yellow, Bl—blue.

TABLE 8.—(*Continued*).

Sources of nitrogen	Parasitic cultures							Saprophytic cultures																
	N-23	79	86	87	104	105	107	89	90	103	106	3369 A *A. flavus*	*A. flavus* (Kr.)	*A. setonii*	A 1	A-12	88-A	88-B	1356 *A. scabies* (?)	3369 B *A. flavus*	3372 *A. viridis*	3374 *A. praecox*	*A. claviger*	*A. tricolor*	*A. xantho-stroma*
Tryptophane, 0.1 per cent																									
Mycelium:																									
Nutritive	4	3	3	3	4	3	4	2	2	2	3	1	2	2	4	2	2	2	2	1	3	3	1	3	2
Aerial	3	1	3	1	3	1	3	0	0	0	1	0	1	2	4	1	0	0	0	0	4	0	0	3	2
Pigmentation	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1BI	0
Tyrosine, 0.1 per cent																									
Mycelium:																									
Nutritive	4	4	4	4	4	5	4	4	3	4	5	3	4	4	5	3	5	4	5	3	5		3	3	5
Aerial	1	2	4	2	2	4	4	2	4	3	4	0	2	3	4	3	4	0	1	0	5		0	4	4
Pigmentation	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1Y	0	0	0	0	0		0	0	0
Blood albumin, 0.5 per cent																									
Mycelium:																									
Nutritive	5	4	4	5	5	5	5	4	4	5	5	5	5	5	5	5	4	4	5	5	5	4	3	5	5
Aerial	2	2	3	3	3	5	4	0	3	5	4	1	2	5	5	5	4	2	5	0	5	3	3	5	5
Pigmentation	3B	2B	4B	4B	2B	3B	2B	0	2B	0	0	0	0	0	0	1B	4B	0	2B	0	2G	0	0	4BIPp	0
Globulin, 0.5 per cent																									
Mycelium:																									
Nutritive	5	5	5	5	5	5	4	4	4	5	5	5	5	5	5	5	4	4	5	5	5	5	4	5	5
Aerial	3	4	2	5	5	5	3	0	4	5	4	2	2	5	4	5	5	2	5	0	5	2	2	5	5
Pigmentation	2B	4B	4B	0	2B	3B	2B	0	3B	0	0	1B	2Y	0	0	1B	4B	0	3B	0	2G	0	0	5BI	0
Urea, 0.5 per cent																									
Mycelium:																									
Nutritive	0	0	0	0	0	4	0	3	0	5	5	4	3	5	0	5	0	0	4	4	0	4	3	5	4
Aerial	0	0	0	0	0	2	0	0	0	5	4	4	0	5	0	5	0	0	0	0	0	4	1	3	4
Pigmentation	0	0	0	0	0	2B	0	0	0	0	0	0	0	0	0	3P	0	0	0	0	0	0	0	2BI	0

TABLE 9.—*Growth of Actinomyces on media containing urea.*[1]

Urea	Parasitic cultures							Saprophytic cultures																	
	N-23	79	86	87	104	105	107	89	90	103	106	3369 A *A. flavus*	*A. flavus* (Kr.)	*A. setonii*	A-1	A-12	88-A	88-B	1356 *A. scabies* (?)	3369-B *A. flavus*	3372 *A. viridis*	3374 *A. praecox*	*A. clavifer*	*A. vinicolor*	*A. xanthostroma*
0.5 per cent urea (added to medium before sterilization) [2]																									
Mycelium:																									
Nutritive	0	0	0	0	0	3–4	0	3	0	5	5	4	3	5	0	4	0	0	5	4	0	4	5	5	1
Aerial	0	0	0	0	0	4	0	0	0	4	0	3	0	4	0	5	0	0	5	0	0	0	4	5	0
Pigmentation	0	0	0	0	0	1B	0	0	0	1B	1B	1B	0	1B	0	3P	0	0	2B	0	0	0	1B	4Bl	0
pH of medium	9 4	9 4	9 4	9 4	9 4	9 2	9 4	9 3	9 4	9 3	9 3	9 2	9 2	9 1	9 4	9 2	9 4	9.4	9 0	9 3	9 4	9 3	9 1	9 0	9 4
Crystals [3]	4	4	4	4	4	1	4	0	4	1	2	2	4	2	4	1	4	4	1	0	4	2	2	1	4
0.5 per cent urea (urea added to medium after sterilization) [2]																									
Mycelium:																									
Nutritive	0	0	0	0	1	0	0	4–3	0	5	5	3–4	2	5	0	5	0	0	5	4–3	0	4	4	5	2–1
Aerial	0	0	0	0	0	0	0	4	0	4	4–3	0	0	3	0	5	0	0	5	0	0	3	3	5	0
Pigmentation	0	0	0	0	0	0	0	0	0	3Y	0	0	0	2YB	0	3YP	0	0	2Y	0	0	0	0	4Bl	0
pH of medium	9 4	9 3	9 4	9 4	9 3	9 4	9 3	9 3	9 4	9 0	9 4	9 3	9 1	9 2	9 4	9 0	9 4	9 4	9 1	9 3	9 3	9 3	9 0	9 0	9 3
Crystals [3]	4	4	4	4	4	4	4	1	4	1	1	4	4	2	4	2	4	4	1	2	4	2	2	1	4
0.5 per cent of urea and 0.3 per cent of potassium nitrate (urea added to medium after sterilization) [2]																									
Mycelium:																									
Nutritive	0	0	0	0	0	4	0	5	0	4	5	3	2	4	0	5	0	0	5	4	0	3	4	5	1
Aerial	0	0	0	0	0	3	0	4	0	4	4	0	0	4–1	0	5	0	0	5	0	0	3	4	5	0
Pigmentation	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4R	0	0	0	0	0	0	0	4Bl	0
pH of medium	9 4	9 4	9 4	9 4	9 4	9 2	9 4	9 3	9 3	9 1	9 3	9 0	9 4	9 3	9 4	9 1	9 3	9 2	9 0	9 0	9 4	9 3	9 3	9 2	9 4
Crystals [3]	4	4	4	4	4	1	4	1	4	1	1	2	2	2	4	1	4	4	1	1	4	2	2	0	4

At the end of the experiment ammonia was present in abundance in all cultures. The amount of growth and intensity of pigmentation are designated by the following numerals: 0—none, 1—very slight, 2—slight, 3—fair, 4—good and 5—very good. Pigments are indicated by letters: B—brown, P—pink, Bl—blue, Y—yellow, R—red.

[2] Two numerals are used to show the variation in growth of cultures in the duplicate test tubes.

[3] The amount of crystals present in these media is designated as follow: 0—none, 1—very slight, 2—slight, 3—good and 4—very good.

Blood albumin.—The majority of cultures grew well on this medium, developing extensive nutritive and aerial mycelia. All the parasitic cultures and a few of the saprophytic developed soluble pigments of different colors. Except for this pigmentation, no significant differences existed between the two groups.

Globulin.—Growth of the cultures on this medium was similar to that on the medium containing blood albumin.

Urea.—Only one parasite culture, 105, produced any growth, but thirteen of the eighteen saprophytic cultures produced extensive growth. Cultures A-12, 105, and *A. tricolor* developed pigmentation.

Occasional observations and tests indicated that the reaction of the urea medium at the end of the experiment was highly alkaline. It was also noted that crystals were present mainly in the test tubes where growth was absent. In order to explain this and also to determine why parasitic Actinomyces failed to grow on this medium, a more detailed experiment with urea was conducted. This was performed in three separate tests.

The first was a duplication of the original experiment with urea. In the second, the same kind of medium was again used but the urea was first sterilized in dry heat at 105° C. before it was added to the slightly warm sterile medium. This was done to determine possible inhibition of growth due to an accumulation of ammonia as a result of decomposition of urea when it was sterilized in an autoclave. This same method was repeated for the third test and 0.3 per cent potassium nitrate was added to the medium as an additional source of nitrogen. This was added to determine whether the lack of growth of the parasitic Actinomyces was due to their inability to use urea or its products of decomposition as sources of nitrogen, or to the toxicity of urea.

Duplicate test tubes of these three media were used for each one of the twenty-five cultures, and at the beginning of the experiments the reaction of the media was adjusted to pH 7.0. The cultures were grown for one month. The final readings are presented in Table 9.

The results of these three tests were practically identical and confirmed the results obtained in the original experiment. In all cases the final reaction of the media, determined colorimetrically with thymol blue, was above pH 9.0. In general, the media on which no growth occurred were more highly alkaline than the media where growth was present. Ammonia, determined with Nessler's reagent, was present in excess in all cases. The amount of crystals present in the medium showed an inverse correlation to the amount of growth produced.

Discussion.—Undoubtedly ammonia was produced as a result of decomposition of urea, and an increase in alkalinity can be attributed to this. Since the same growth was obtained on a urea medium containing potassium nitrate as was obtained without it, the failure of certain cultures to develop growth cannot be attributed to their inability to use urea as a source of nitrogen, but to other factors, possibly the toxicity of urea itself or of the products of its decomposition. Since fewer crystals were

present in media in which more growth appeared, it is possible that some of the substances of the media, which were extensively used by the cultures, entered into the formation of these crystals. Since the reaction of the media in which growth occurred was less alkaline than where it did not develop, it is probable that an excess of ammonia was present in the latter case. Analysis [3] proved that these crystals were $Mg(NH_4)PO_4 \cdot 6H_2O$, thereby confirming the assumptions made.

When water acts upon urea, carbon dioxide and ammonia are liberated as products of decomposition. If, then, the real cause of inhibition of growth on this medium is a product of the decomposition of urea, it is evident that ammonia is the only substance which could induce this. Again a question arises as to whether the inhibitory action of ammonia is due to its direct toxicity or to the high alkalinity it produces. In either case, ammonia is probably the substance which inhibited the growth of all the parasitic and of some of the saprophytic Actinomyces.

Previous investigators have reported on the toxic effect of ammonia on a number of organisms. Boas (5) reported that when *Aspergillus niger* was grown on a nutrient medium containing 5 per cent maltose and 2 per cent urea, it produced such quantities of ammonia as to cause the death of the organism. Other fungi, such as *Botrytis cinerea* and *Oidium,* when grown under similar conditions remained alive for months. Practically the same results were obtained by Pratt (25, 26), when she grew different fungi on a medium similar to that which Boas used. These experiments indicated that ammonia has a selective effect, and proves to be highly toxic to some organisms, while to others it probably is even beneficial because it serves as a source of nitrogen. In recent publications by Neal (23) and Streets (31), the former reports that ammonia was toxic to *Phymatotrichum omnivorum* in pure cultures, while the latter reports that he obtained successful control of the same fungus in infected soils by using ammonium sulphate and ammonium hydroxide. Leach and Davey (16) found that ammonia was toxic to *Sclerotium rolfsii* and that when ammonium sulphate or ammonium hydroxide was dissolved in irrigation water (approximately 300 parts per million of ammonia), the percentage of infection on sugar beets by this organism was reduced significantly.

INHIBITION OF ACTINOMYCES BY AMMONIUM COMPOUNDS, UREA, POTASSIUM BICARBONATE, AND CALCIUM HYDROXIDE

To obtain more information on the toxicity of ammonia to parasitic Actinomyces, the following ammonium-evolving compounds were tested: ammonium carbonate, ammonium bicarbonate, ammonium hydroxide, and urea. In addition, the toxicity of potassium bicarbonate to Actinomyces was tested mainly because Pratt (25, 26), who made intensive studies of the "staling" of *Fusarium* and *Botrytis* growing on Richards'

[3] The author is indebted to Dr. F. T. Donaldson of Montana State College for the analysis of these crystals.

solution, concluded that it was caused by an accumulation of potassium bicarbonate. Since the compositon of Richards' solution was similar to that of the medium used in this experiment, it was thought that potassium bicarbonate might be a factor in these tests. Pratt also reported the presence of ammonia in her medium, which she concluded was produced as a result of metabolism of the cultures, because nitrogen was added only in the form of potassium nitrate. She reported the presence of $Mg(NH_4)PO_4 \cdot 6H_2O$ crystals in her medium.

The possibility of toxicity of calcium hydroxide in a cultural solution was also tested in this study. Blodgett and Cowan (4) were able to reduce the amount of potato scab by applying large amounts of calcium hydroxide to a soil inoculated with *A. scabies*. They believed that this occurred because of an increase in the alkalinity of the soil.

All of the compounds were added to the basic liquid medium as before (see page 19), and in amounts to make the following molecular solutions: M/1,000, M/100, M/50, and M/25. The four solutions containing nitrogenous compounds were used alone and in combination with 0.3 per cent of potassium nitrate as an additional source of nitrogen, and this same amount of potassium nitrate was also added to media containing compounds of potassium bicarbonate and calcium hydroxide.

The three nitrogenous compounds, ammonium carbonate, ammonium bicarbonate, and ammonium hydroxide, were not sterilized before they were added to the sterilized solutions, because the large amount of free ammonia present indicated that they were sterile. Urea, potassium bicarbonate, and calcium hydroxide were sterilized in dry heat at 105° C. before they were added to the sterilized media. The reaction of the media at the beginning of the experiment was determined by the colorimetric method and at the end by the electrometric method with a glass electrode. The two saprophytic Actinomyces, *A. setonii* and A-12, and the two parasitic Actinomyces, N-23 and 107, were grown on 50 cc. of each solution in 125 cc. Erlenmeyer flasks. The inoculum was added in the form of a suspension of spores and mycelium at the rate of 1 cc. per flask. Two flasks containing the same kind of medium (0.3 per cent potassium nitrate as a source of nitrogen) but without additional nitrogenous compounds, were used as checks for each organism. Cultures were grown for about seven weeks. At the end of this period, readings were taken of the amount of growth, and at the same time qualitative determinations were made of the presence of nitrates, nitrites, and ammonia in the solutions. The presence of ammonia was determined with Nessler's reagent, and the presence of nitrates and nitrites with sulphanilic acid and alpha-naphthylamine, as described by Fred and Waksman (10).

This test was designed to investigate the effects of the compounds on the growth of Actinomyces, and the possible correlation of toxicity with the reaction of the media. The results are presented in Table 10.

The growth and behavior of the organisms will be discussed separately for each of the various compounds. For convenience, the medium

TABLE 10.—*Toxicity of different chemical substances to parasitic and saprophytic cultures of Actinomyces.*[1]

Compound used	Strength of solution			*A. setonii* (saprophytic)						A-12 (saprophytic)					
	Molecular	Gm. per liter	pH	Nu. myc.	Aer. myc.	Final pH	NO_3	NO_2	NH_3	Nu. myc.	Aer. myc.	Final pH	NO_3	NO_2	NH_3
$(NH_4)_2CO_3$	M/1000	0.114	6.9	5	4	5 12	0	0	.	4	4	5.65	0	0	.
	M/100	1.14	8.7	4	4	7.18	0	0	....	3	3	5.54	0	0	..
	M/50	2.28	9.0	3	3	7 15	0	0	....	3	2	6.13	0	0	....
	M/25	4.56	9.3	0	0	7.28	0	0	....	0	0	8.03	0	0	....
$(NH_4)_2CO_3$ + KNO_3	M/1000	0.114	6.9	5	5	8.56	..	...	...	5	5	8.66	..	0	...
	M/100	1.14	8.6	4	4	7.68	...	..	....	3	3	6.12	...	0	..
	M/50	2.28	9.0	2	3	6.55	....	..	....	2	1	6.65	....	..	....
	M/25	4.56	9.2	0	0	7.68	....	..	....	0	0	7.85	....	..	....
NH_4HCO_3	M/1000	0.079	6.7	5	4	6.02	0	0	.	4	3	6.14	0	0	.
	M/100	0.79	8.2	5	4	6.88	0	0	...	3	3	5.64	0	0	..
	M/50	1.58	8.5	5	4	7.81	0	0	....	3	3	5.52	0	0	....
	M/25	3.16	8.6	4	4	7.60	0	0	....	3	2	5.81	0	0	....
NH_4HCO_3 + KNO_3	M/1000	0.079	6.7	5	5	8.78	..	...	..	5	5	8.93	..	0	.
	M/100	0.79	8.1	4	5	8.11	....	..	....	5	5	8.50	...	0	..
	M/50	1.58	8.4	5	4	7.82	....	..	....	4	3	5.18	....	0	....
	M/25	3.16	8.5	3	3	6.65	....	..	....	2	2	5.85	....	0	....
NH_4OH	M/1000	0.035	6.8	4	4	5.17	0	0	0	4	3	5.26	0	0	.
	M/100	0.35	8.5	4	4	5.92	0	0	.	4	4	5.43	0	0	.
	M/50	0.7	9.6	4	4	7.28	0	0	....	4	2	5.50	0	0	...
	M/25	1.4	9.8	2	0	6.00	0	0	....	4	3	5.78	0	0	....
NH_4OH + KNO_3	M/1000	0.035	6.8	5	5	8.92	0	...	..	5	5	8.75	..	0	.
	M/100	0.35	8.5	5	5	8.48	....	..	...	4	5	7.30	..	..	.
	M/50	0.7	9.5	5	5	5.78	....	0	...	3	1	5.85	....	0	...
	M/25	1.4	9.8	5	5	5.90	....	0	....	3	1	5.95	....	0	....
$(NH_2)_2CO$	M/1000	0.06	6.5	5	5	5.66	0	0	.	5	4	5.40	0	0	.
	M/100	0.6	6.5	5	5	7.90	0	0	....	5	4	8.22	0	0	...
	M/50	1.2	6.5	5	5	8.02	0	0	....	5	4	8.40	0	0	....
	M/25	2.4	6.5	5	5	8.25	0	0	....	5	4	8.48	0	0	....
$(NH_2)_2CO$ + KNO_3	M/1000	0.06	6.4	5	5	8.81	..	...	.	5	5	8.80	.	.	.
	M/100	0.6	6.4	5	5	8.10	...	..	....	4	4	8.28	...	0	...
	M/50	1.2	6.4	5	5	8.05	....	..	....	4	3	8.28	...	0	....
	M/25	2.4	6.4	5	5	8.05	....	..	....	4	4	8.12	...	0	....
$KHCO_3$ + KNO_3	M/1000	0.1	6.7	4	3	8.78	.	..	.	3	4	8.30	..	.	.
	M/100	1.0	8.1	4	3	8.90	..	..	.	4	5	8.54	..	.	.
	M/50	2.0	9.3	4	3	9.04	..	..	.	3	3	9.45	...	...	.
	M/25	4.0	9.6	3	3	9.24	..	..	.	2	0	8.90	....	...	.
$Ca(OH)_2$ + KNO_3	M/1000	0.074	6.9	5	5	8.81	.	...	.	4	4	8.98	.	..	.
	M/100	0.7	8.5	4	1	6.26	...	...	.	3	3	7.70	..	.	.
	M/50	1.4	9.6	3	3	8.26	...	...	.	3	3	8.95	.	...	.
	M/25	2.8	9.6	2	2	8.46	....	...	.	0	0	8.05	.	...	.
Check	1		7.0	4	4	8.48	.	.	..	4	4	9.10	..	..	.
Check	2		7.0	4	4	8.42	.	.	..	3	3	9.10	..	..	.

[1] The amount of growth is designated by the following numerals: 0—none, 1—very slight, 2—slight, 3 fair, 4—good, and 5—very good. The amounts of nitrates, nitrites, and ammonia in these solutions, from none to the greatest amount, are designated as: 0, ., , , . .

TABLE 10.—(*Concluded*).

Compound used	Strength of solution			N-23 (parasitic)						107 (parasitic)					
	Molecular	Gm. per liter	pH	Nu. myc.	Aer. myc.	Final pH	NO_3	NO_2	NH_3	Nu. myc.	Aer. myc.	Final pH	NO_3	NO_2	NH_3
$(NH_4)_2CO_3$	M/1000	0.114	6.9	4	4	6.35	0	0	.	4	3	6.25	0	0	.
	M/100	1.14	8.7	0	0	7.41	0	0	....	0	0	7.35	0	0	....
	M/50	2.28	9.0	0	0	7.42	0	0	....	0	0	7.35	0	0	....
	M/25	4.56	9.3	0	0	7.35	0	0	...	0	0	7.72	0	0	....
$(NH_4)_2CO_3$ + KNO_3	M/1000	0.114	6.9	1	0	6.05	....	...	0	1	1	6.02	....	....	..
	M/100	1.14	8.6	0	0	7.35	....	.	....	0	0	7.35	....	.	....
	M/50	2.28	9.0	0	0	7.60	....	.	....	0	0	7.55	....	.	....
	M/25	4.56	9.2	0	0	7.82	....	.	....	0	0	7.90	....	.	....
NH_4HCO_3	M/1000	0.079	6.7	4	4	5.55	0	0	.	4	3	6.15	0	0	.
	M/100	0.79	8.2	3	4	5.40	0	0		3	3	7.05	0	0	...
	M/50	1.58	8.5	2	3	6.70	0	0	....	2	0	7.32	0	0	....
	M/25	3.16	8.6	0	0	7.70	0	0	....	0	0	7.42	0	0	....
NH_4HCO_3 + KNO_3	M/1000	0.079	6.7	1	0	5.82	....	....	..	1	0	5.72	....	....	..
	M/100	0.79	8.1	2	0	6.50	....	....	...	2	0	6.65	....	....	...
	M/50	1.58	8.4	2	0	7.10	....	....	....	2	0	7.18	....	....	....
	M/25	3.16	8.5	0	0	7.60	....	..	....	0	0	7.90	....	..	....
NH_4OH	M/1000	0.035	6.8	4	4	6.30	0	0	.	4	3	6.00	0	0	.
	M/100	0.35	8.5	2	3	5.81	0	0	.	3	0	6.20	0	0	.
	M/50	0.7	9.6	0	0	7.35	0	0	....	0	0	7.05	0	0	....
	M/25	1.4	9.8	0	0	7.50	0	0	....	0	0	7.45	0	0	....
NH_4OH + KNO_3	M/1000	0.035	6.8	1	0	7.91	....	....	..	2	1	7.85	...	....	..
	M/100	0.35	8.5	2	0	6.20	....	....	...	2	0	7.03	....	....	...
	M/50	0.7	9.5	0	0	7.40	....	..	....	0	0	7.30	....	..	....
	M/25	1.4	9.8	0	0	7.40	....	..	....	0	0	7.34	....	..	....
$(NH_2)_2CO$	M/1000	0.06	6.5	4	4	6.40	0	0	.	4	4	6.30	0	0	.
	M/100	0.6	6.5	4	3	8.25	0	0	....	4	3	8.18	0	0	...
	M/50	1.2	6.5	2	1	7.69	0	0	....	4	0	8.38	0	0	....
	M/25	2.4	6.5	2	3	7.72	0	0	....	3	1	8.09	0	0	....
$(NH_2)_2CO$ + KNO_3	M/1000	0.06	6.4	1	1	5.94	..	....	0	2	2	6.04	....	....	.
	M/100	0.6	6.4	2	0	8.10	...	...	....	3	0	7.86	....	....	....
	M/50	1.2	6.4	1	0	8.08	....	....	....	2	0	7.74	....	....	....
	M/25	2.4	6.4	1	0	8.48	....	....	....	1	0	8.25	....	....	....
$KHCO_3$ + KNO_3	M/1000	0.1	6.7	2	1	6.02	..	....	.	2	1	5.90	....	...	.
	M/100	1.0	8.1	3	0	7.01	...	....	.	2	0	7.35	....	....	.
	M/50	2.0	9.3	2	0	8.50	....	....	.	2	0	8.46	....	....	.
	M/25	4.0	9.6	0	0	8.80	....	...	.	0	0	8.85	..	..	.
$Ca(OH)_2$ + KNO_3	M/1000	0.074	6.9	3	4	6.20	...	....	.	3	0	5.70	....	....	.
	M/100	0.7	8.5	3	2	7.95	....	....	.	2	3	8.00	....	....	.
	M/50	1.4	9.6	0	0	8.00	....	...	.	0	0	8.02	..	.	.
	M/25	2.8	9.6	0	0	8.00	....	...	.	0	0	8.00	.	.	.
Check	1		7.0	3	2	5.60	....	....	.	4	3	5.60	....	...	
Check	2		7.0	3	1	5.60	....	....	.	2	2	5.78			.

lacking potassium nitrate will be designated as A and the one containing potassium nitrate as B. In all the tests, the two parasitic cultures were similar in their growth and behavior, as were the two saprophytic cultures. The two groups were, however, entirely different from each other.

AMMONIUM CARBONATE

Saprophytic cultures *A. setonii* and A-12.—Growth was fair in the three solutions M/1,000, M/100, and M/50 of media A and B, but no growth was found in solution M/25. At the start of the experiment, the reaction of both media was progressively more alkaline, with an increase in concentration of ammonium carbonate. The final reaction of the low concentrations of media A and B became acid, probably because of metabolism of the cultures. The high concentrations showed an alkaline reaction which was considerably lower than that at the beginning of the experiment. This undoubtedly was caused by decomposition of ammonium carbonate.

Approximately the same results were obtained for the final reaction of all solutions of medium B, with the exception of solution M/1,000, which showed a considerable increase in alkalinity. Probably this occurred because only a very small amount of ammonium carbonate was present, and consequently the cultures used nitrogen from potassium nitrate almost exclusively. For this reason the reaction was shifted toward the alkaline side. Waksman and Joffe (34) have stated that when Actinomyces reduced nitrates to nitrites, "the oxygen split off from the nitrate molecule is united with the hydrogen or other reducing substances of the medium, thus tending to reduce the hydrogen tension of the medium." Determinations of nitrates and nitrites at the end of the experiment showed that in the M/1,000 solution, nitrates were reduced more than in the others. This indicated that the organisms could use ammonium nitrogen just as well as nitrogen from nitrates. Culture A-12 showed a greater ability to assimilate nitrites than *A. setonii*. This ability was also observed in a later experiment with tyrosine (Tables 16, 17, and 20). Ammonia was present in abundance in all solutions except M/1,000.

Parasitic cultures N-23 and 107.—Growth occurred only in solution M/1,000 of medium A, and there was only a trace of growth in the same solution of medium B. The pH values of all solutions of both media A and B were considerably lower than at the beginning of the experiment. Only small amounts of nitrates were reduced to nitrites in all solutions of medium B. Ammonia was present in abundance in all solutions except M/1,000.

The outstanding difference between the two saprophytic and two parasitic cultures was that only the M/25 solution of ammonium carbonate was fatal to the saprophytic cultures, while the weaker solutions M/100 to M/25 inclusive were fatal to the parasitic cultures. It is the author's belief that the failure of the parasitic cultures to grow in these solutions was due to the direct toxicity of ammonia and not to the high alkalinity of the medium at the beginning of the experiment.

AMMONIUM BICARBONATE

Saprophytic cultures *A. setonii* and A-12.—Both cultures grew relatively well in all solutions of ammonium bicarbonate of media A and B. The final pH values of all solutions of both media were considerably lower than those at the beginning of the experiment, except in solutions M/1,000 and M/100 of medium B, where they were higher. Probably the same explanation is applicable here as in the experiment with ammonium carbonate. Determinations of nitrates, nitrites, and ammonia at the end of the experiment showed approximately the same results as in the experiment with ammonium carbonate.

Parasitic cultures N-23 and 107.—Slight growth occurred in the three solutions M/1,000, M/100, and M/50 of medium A and only very slight growth in the same solutions of medium B. Growth was entirely absent in solutions M/25 of both media A and B. The final reaction of both media was slightly acid in low concentrations and slightly alkaline in high concentrations of ammonium bicarbonate. There was considerable reduction of nitrates to nitrites in medium B. The amount of residual ammonia increased with an increase in concentration of ammonium bicarbonate.

Both saprophytic cultures grew relatively well but the parasitic cultures developed slight growth only at low concentrations and no growth at all in concentration M/25. From this experiment, it is concluded that ammonium bicarbonate is toxic to parasitic cultures but its toxicity is less than that of ammonium carbonate. Probably this is due to the fact that ammonium bicarbonate has considerably less ammonia than ammonium carbonate.

AMMONIUM HYDROXIDE

Saprophytic cultures *A. setonii* and A-12.—Both cultures grew abundantly in most solutions of media A and B. The final pH values of solutions M/1,000 and M/100 of medium B were higher than those of the other two solutions, which is in agreement with the results of the previous experiments. Determinations of nitrates, nitrites, and ammonia at the end of the experiment showed approximately the same results as in the two previous experiments.

Parasitic cultures N-23 and 107.—Slight growth was present in solutions M/1,000 and M/100 of ammonium hydroxide of medium A, while only traces of growth were present in the same solutions of medium B. The final pH values of practically all solutions were considerably lower than they were at the beginning of the experiment. Determinations of nitrates and nitrites in medium B showed that although traces of growth were present, considerable amounts of nitrates were reduced to nitrites. The amount of residual ammonia was present in proportion to the original concentration of ammonium hydroxide.

In this test, the saprophytic Actinomyces grew well on both types of media but the parasitic cultures developed growth only in the two lowest concentrations of these media. It is evident that solutions M/25 and M/50

of ammonium hydroxide are toxic to the parasitic cultures. More growth was developed in this test than in that with ammonium carbonate, probably because proportionally less ammonia was present.

UREA

Saprophytic cultures *A. setonii* and A-12.—Both organisms grew well in all concentrations of urea with both media. At the start the reaction of the solutions was slightly acid and at the end all solutions except M/1,000 of medium A became alkaline, probably because of the ammonia liberated from the urea. The high alkalinity of the media at the end of the experiment indicated that decomposition of urea took place more slowly than for the nitrogenous compounds previously discussed. Reduction of nitrates was much more extensive at a low than at a high concentration of urea. Culture A-12 used practically all the nitrites produced from nitrates. The amount of residual ammonia was present in proportion to the amount of urea.

Parasitic cultures N-23 and 107.—Both cultures developed a fair amount of growth in all solutions of medium A and only a slight amount in solutions of medium B, showing a tendency to produce less growth with an increase in concentration of urea. The final reaction of all solutions of both media except solutions M/1,000 was alkaline. Pronounced reduction of nitrates to nitrites took place. The amount of residual ammonia was in proportion to the amount of urea.

The saprophytic organisms developed abundantly, while the parasitic cultures produced only a fair to a very slight amount of growth. Low concentrations of urea apparently do not produce sufficient amounts of ammonia to inhibit growth of parasitic cultures. In a previous experiment in which 0.5 per cent of urea was used, the amount of ammonia produced was sufficient to completely inhibit the growth of parasitic Actinomyces. In the present test the highest amount of urea was in solution M/25, which was equivalent to only 0.24 per cent of urea and approximately one-half the concentration used previously. Even with these low concentrations, there was an increasing inhibition of growth as the concentration of urea was increased.

POTASSIUM BICARBONATE

Saprophytic cultures *A. setonii* and A-12.—Both cultures grew fairly well in all solutions of this medium except culture A-12 at M/25. The final reaction of the two lowest concentrations of potassium bicarbonate became more alkaline, probably as a result of metabolism and the other two concentrations except M/50 of culture A-12 showed a decrease in alkalinity.

Parasitic cultures N-23 and 107.—Growth was only slight with the three solutions M/1,000, M/100, and M/50 and no growth developed in solution M/25. The pH values of all solutions at the end of the experiment were lower than those at the beginning.

A pronounced reduction of nitrates occurred with both groups of organisms, and traces of ammonia were present. From this test it is concluded that potassium bicarbonate inhibits the growth of parasitic cultures more than that of saprophytic cultures.

CALCIUM HYDROXIDE

Saprophytic cultures *A. setonii* and A-12.—Growth was fairly good at low concentrations of calcium hydroxide, but at high concentrations it was noticeably less. Culture A-12 failed to grow in solution M/25. Solution M/1,000 showed a pronounced increase in alkalinity.

Parasitic cultures N-23 and 107.—Growth of both cultures in the two lowest concentrations was only fair and none developed in the two highest concentrations. At the end of the experiment all solutions showed a proportional decrease in pH values.

A pronounced reduction of nitrates took place with both parasitic and saprophytic cultures, and traces of ammonia were present. The two highest concentrations of calcium hydroxide retarded the growth of saprophytic cultures and entirely inhibited the growth of parasitic cultures. It was not known whether this inhibition of growth of parasitic cultures was due to the high degree of alkalinity developed or to the direct toxicity of the calcium hydroxide.

Checks.—Both saprophytic cultures developed good growth on the check medium but the parasitic cultures produced only a fair amount. The final reaction of the medium on which saprophytes grew became highly alkaline, but it became acid in the case of the parasites. The saprophytic cultures reduced nitrates more extensively than the parasitic cultures. A slight amount of ammonia was present in both cases.

CONCLUSIONS

Throughout this experiment, it was found that growth and behavior of the two saprophytic cultures were very similar, and that the two parasitic cultures also resembled each other but that the two groups differed greatly. It is evident that the physiologies of the two are quite different. It is believed that in the case of all the nitrogenous compounds tested, the inhibition of growth of parasitic cultures was due to the evolution of ammonia from these compounds.

It might be considered that the high initial alkalinity of the solutions inhibited the growth of parasitic Actinomyces, but a comparison of the initial reaction of the solutions shows that some solutions in which no growth was present had approximately the same pH as other solutions with lower concentrations of the tested substances where growth appeared. For example, both parasitic cultures developed a slight amount of growth in solution M 50 of ammonium bicarbonate which had an initial pH 8.5, while no growth was present in solution M/25 of the same substance with an initial pH of 8.6. Approximately the same situation developed in these same solutions in which ammonium bicarbonate was combined with

potassium nitrate. The author believes that a slight variation in the reaction of the media could not have such a pronounced influence on the growth of these cultures.

In testing the different concentrations of potassium bicarbonate and calcium hydroxide, the situation was about the same as stated above. It is not known whether the inhibition of growth of parasitic cultures in the case of these two compounds was due to high alkalinity of the media or to their direct toxicity. For example, culture N-23 showed a fair amount of growth in solution M/100 of calcium hydroxide, where the final pH was 7.95, and no growth at all in solution M/50, which had a final pH 8.0. Practically the same results were obtained with culture 107. Such a small difference in alkalinity probably would not have such a significant influence on the growth of Actinomyces. It is possible that in some instances the high alkalinity at the beginning of the experiment was fatal to the parasitic cultures. In a solution of M/50 of potassium bicarbonate in which the initial pH was 9.3, slight growth was developed by both of the parasitic cultures, while in solutions M/25 of potassium bicarbonate, M/50 and M/25 of calcium hydroxide, with the initial pH at 9.6, no growth appeared for either of the parasitic cultures. It is possible that either a slight increase in alkalinity above a pH 9.3 was fatal to the parasitic Actinomyces, or inhibition of growth was due to the toxic effect of these substances and not to the reaction of the medium.

As previously noted, Blodgett and Cowan (4) in their work found that with increasing amounts of calcium hydroxide added to soil inoculated with *A. scabies,* the amount of scab was diminished. In conclusion they stated that "no reason was found for believing that lime has any effect on potato scab other than through its effect on the alkalinity of the soil." From the results of the author's tests, it would appear that while a high degree of alkalinity has a suppressive effect on the development of parasitic Actinomyces it is possible that calcium hydroxide has also a direct toxic effect on these organisms.

EFFECT OF HIGH ALKALINITY ON ACTINOMYCES

In order to obtain more information concerning the effect of high alkalinity on the growth of Actinomyces, one additional test was conducted. An agar medium of the same basic composition with 0.3 per cent of potassium nitrate as a source of nitrogen was used as before, and divided into three parts. The reaction of each medium was adjusted to pH 9.5 with sodium hydroxide, potassium hydroxide and calcium hydroxide. Three sets of duplicate test tubes containing these media were inoculated with the three saprophytic cultures A-12, 3374 (*A. praecox*), and *A. setonii,* and the three parasitic cultures N-23, 86, and 107, and allowed to grow for approximately three weeks. During this time several readings of growth were taken. Final readings are presented in Table 11.

Growth of all the saprophytic cultures was visible on the second day following inoculation, while growth of the parasitic cultures did not

TABLE 11.—*The effect of high alkalinity on the growth of Actinomyces.*[1]

Media	Saprophytic cultures			Parasitic cultures			Check
	A-12	3374 *A. praecox*	*A. setonii*	N-23	86	107	
Media adjusted to pH 9.5 with:							
$Ca(OH)_2$							
Mycelium:							
Nutritive	5	5	5	5	4	4	0
Aerial	5	5	5	4	3	4	0
Final pH.....	9 2	9 0	9 0	9 0	8 8	8.8	8 5
NaOH							
Mycelium:							
Nutritive	5	5	5	5	4	4	0
Aerial	5	5	5	3	0	2	0
Final pH.....	9.2	9.2	9.0	8.6	8.8	8.9	8 5
KOH							
Mycelium:							
Nutritive	5	5	5	5	5	4	0
Aerial	5	5	5	3	0	2	0
Final pH.....	9.0	8 8	9 0	9.0	8.8	8 8	8 5

[1] Amount of growth is designated by the following numerals: 0—none, 1—very slight, 2—slight, 3—fair, 4—good, and 5—very good.

begin until the fifth day after inoculation and progressed thereafter at about the same rate as the growth of the saprophytic cultures. Final readings showed that the parasitic cultures developed slightly less nutritive mycelium and considerably less aerial mycelium than the saprophytic cultures. In general there was little apparent difference in the amount of growth of the two groups of organisms. The final pH of the media, determined colorimetrically, was 8.8 to 9.2 for saprophytic cultures, and 8.6 to 9.0 for parasitic cultures.

In comparing the growth and initial reaction of the three media in this experiment with the growth and initial reaction in solutions M/50 and M/25 of calcium hydroxide in the previous experiment, good growth developed on the media in the present experiment and none in the previous one, although all media had approximately the same initial reaction. It is evident that a high alkalinity has a more suppressive effect on parasitic cultures than on saprophytic cultures, and at the same time the results of this experiment showed that the parasitic cultures were able to shift the isoelectric point of a medium with a high degree of alkalinity to a more favorable point.

The results of this experiment indicate that the reaction of the media is not the critical point in the growth of parasitic cultures, but that the toxicity of the compounds used is the inhibiting factor.

EFFECT OF UREA ON INFECTION OF POTATO TUBERS

When it was found that 0.5 per cent of urea added to a medium inhibited the growth of parasitic Actinomyces, tests were conducted in a greenhouse to determine if urea would control scab of potatoes. The same type of 7-inch pots containing 7 pounds of soil of the same composition as in the pathogenicity tests were prepared. The tests were divided into

TABLE 12.—*Effect of urea on the development of potato scab (culture N-23).*

Extent of scab	Amount of urea added in each pot before sterilization of soil [1]				Amount of urea added in each pot after sterilization of soil				Check, uninoculated—no urea added
	0.14 gram	0.30 gram	2.00 grams	5.00 grams	0.14 gram	0.30 gram	2.00 grams	5.00 grams	
pH of soil after:									
Sterilization	6 0	6 0	6 3	6 5	6 2	6 4	6 7	7 2	6.0
2 weeks	7.0	7.1	7 5	8 0	7 0	7 1	7 3	8 4	6 8
Digging	7.7	7.7	7.5	6 9	7.7	7.7	7.0	6 6	7.6
Tubers:									
Number	27	25	36	36	39	27	17	33	27
Wt. (*grams*)	900	828	975	850	775	755	510	610	900
Percentage of tuber surface scabby: [2]									
0.....	33 34	36 37	56 42	85.30	38.07	66.23	85.30	100.00	97.23
0– 2.....	44 45	60 60	30 76	8.82	25.81	15.23	9.80	0.00	2.77
2– 25.....	16.16	3 03	7 69	5.88	6.45	8.61	4.90	0.00	0.00
25– 75.....	5.55	0 00	5 13	0.00	9.67	9.93	0.00	0.00	0.00
75–100.....	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

[1] Six 7-inch clay pots used for each set.
[2] Based on weight of tubers.

two parts. To the soil of the first part, urea was added before sterilization, and to the soil of the other part, it was added after sterilization. The second set was used as a check because it was thought that by prolonged heating of the soil in the autoclave, urea might decompose.

Both of these sets were subdivided into four smaller ones of six pots each, according to the amount of urea added. The urea was well mixed with the soil before the pots were filled. Culture N-23 was used for inoculating the soil treated with urea and in which potatoes had been planted. In addition to these eight experimental sets, one additional non-inoculated set without urea was used as a check. An inoculated set lacking urea was not used because from previous experiments it was known that culture N-23, which was used for soil inoculation, was highly pathogenic (Table 1).

The reaction of the soil in the different series was determined at the beginning of the experiment, before and after adding urea. One determination was made two weeks later and another shortly before digging. The colorimetric method was used in making determinations of soil reaction in all cases except the last, where the electrometric method with a glass electrode was used. At the time of maturity the plants were dug and scab readings were taken in the same manner as in the test for pathogenicity. The results of these readings are presented in Table 12.

When urea was added to the soil, either before or after sterilization, the amount of scab was progressively less with increasing amounts of urea, and this was especially evident where urea was added to the soil after sterilization. The set with 2 grams of urea per pot had only traces of scab, while the set with 5 grams of urea per pot had no scab. Thus the decrease in amount of scab was very pronounced. This control of scab very likely was due to the toxic effect of the products of decomposition of urea (ammonia), and not to a change of reaction of the soil.

There were no significant changes in the reaction of the soil throughout the experiment. Control of scab was less evident when urea was added to the soil before sterilization probably because most of the urea was decomposed in the soil at the time of sterilization, and for that reason left only insignificant amounts to produce an effect on the scab organism. In contrast to the above, the set in which urea was added to the soil after sterilization showed that probably decomposition of urea and the development of the scab organism took place at the same time, so that the action of the toxic substance on the scab organism was more pronounced.

This experiment was performed before it was determined that other ammonia-evolving substances, such as ammonium carbonate, ammonium bicarbonate, and ammonium hydroxide, had greater toxic effects on the scab organism than urea (Table 10). Owing to lack of time, similar experiments with other substances were not conducted in a greenhouse or field.

CONCLUSIONS

From these results the different nitrogenous compounds tested can be arranged in the following order according to the ability of Actinomyces to utilize them: globulin, asparagine, blood albumin, sodium nitrate, tyrosine, potassium nitrate, histidine-hydrochloride, phenylalanine, ammonium sulphate, tryptophane, and urea. These results, in general, agree with those of Waksman (33), except that some of the cyclic amino acids used in this test were not as good sources of nitrogen for Actinomyces as the aliphatic amino acids used by him. Another exception noted in the use of urea, was that most of the saprophytic cultures grew on a urea medium rather well, while practically all parasitic cultures failed to grow. This is probably due to the toxic effect of ammonia developed in this medium from the urea.

In experiments in which the toxicity of different ammonia-evolving compounds was tested, it was found that ammonia was highly toxic to parasitic Actinomyces. In addition, the compounds potassium bicarbonate and calcium hydroxide also inhibited the growth of the parasitic cultures considerably more than they did the saprophytic cultures. A urea compound applied to the soil inoculated with parasitic Actinomyces reduced potato scab.

It is hoped that the results of these tests, in which it was found that certain compounds, or products of their decomposition, were highly toxic to parasitic Actinomyces, will aid in developing some practical methods for the control of potato scab.

EFFECT OF CARBON:NITROGEN RATIOS ON THE GROWTH OF ACTINOMYCES

A comparative study was made of the effect of different ratios of carbon to nitrogen on the growth of parasitic and saprophytic Actinomyces grown under similar conditions. A synthetic medium of the following composition was made:

KH_2PO_4	1.0 g.	KCl	0.5 g.	Agar	15.0 g.
$MgSO_4 \cdot 7H_2O$	0.5 g.	$FeSO_4$	0.01 g.	Dist. water	1,000 cc.

Dextrose was added as a source of carbon, and asparagine and potassium nitrate were used in duplicate sets as sources of organic and inorganic nitrogen. Seven ratios of carbon compounds to nitrogen compounds, expressed in grams per liter of medium, were prepared, using dextrose in combination with asparagine or potassium nitrate: 10:0.1, 10:1, 10:5, 10:10, 5:10, 1:10, and 0.1:10.

Three parasitic cultures, N-23, 86, and 107, and three saprophytic cultures, A-12, 3374 (*A. praecox*), and *A. setonii*, were tested in duplicate test tubes containing 8 to 10 cc. of medium. Before sterilization, the reaction of all media was adjusted to pH 7.0. All cultures were grown for one month and during this time several readings of growth were taken, the last of which are presented in Tables 13 and 14.

TABLE 13.—*Comparative growth of parasitic and saprophytic Actinomyces as influenced by the amount of carbohydrates (dextrose) and nitrogen (asparagine) in the medium.*[1]

Grams per liter of medium		N-23 (parasitic)			86 (parasitic)			107 (parasitic)		
Dextrose	Asparagine	Nu. myc.	Aer. myc.	Pigment	Nu. myc.	Aer. myc.	Pigment	Nu. myc.	Aer. myc.	Pigment
10.0	0.1	3	3	0	2	3	0	3	2	0
10.0	1.0	4	5	0	4	4	0	4	4	0
10.0	5.0	4	3	1 B	4	0	1 B	4	3	1 B
10.0	10.0	5–4 [2]	0	2 B	5–4	0	3 B	5–4	0	3 B
5.0	10.0	5	0–1	0	5	0	0	5	1	0
1.0	10.0	3–4	1	0	4	0	0	4	1	0
0.1	10.0	3	0	0	3	0–1	0	3	1–2	0

Grams per liter of medium		A-12 (saprophytic)			3374 (*A. praecox*) (saprophytic)			*A. setonii* (saprophytic)		
Dextrose	Asparagine	Nu. myc.	Aer. myc.	Pigment	Nu. myc.	Aer. myc.	Pigment	Nu. myc.	Aer. myc.	Pigment
10.0	0.1	3	2	1 P	4	2	0	4	3	0
10.0	1.0	5	4	1 P	5	4	0	5	5	0
10.0	5.0	5	5	2 P	5	4	0	5	5	0
10.0	10.0	5	5	3 P	5	4	0	5	5	0
5.0	10.0	5	5	0	5	5	0	5	5	0
1.0	10.0	4	4	0	5	4	0	5	5	0
0.1	10.0	3	3	0	5	4	0	5	5	0

[1] The amount of growth and intensity of pigmentation in Tables 13 and 14 are designated by the following numerals: 0. 1. 2. 3. 4. and 5. The color is indicated by letters: B—brown. P—pink.
[2] Two numerals are used to show the variation in growth of cultures in the duplicate test tubes.

TABLE 14.—*Comparative growth of parasitic and saprophytic Actinomyces as influenced by the amount of carbohydrates (dextrose) and nitrogen (KNO_3) in the medium.*[1]

Grams per liter of medium		N-23 (parasitic)			86 (parasitic)			107 (parasitic)		
Dextrose	KNO_3	Nu. myc.	Aer. myc.	Pigment	Nu. myc.	Aer. myc.	Pigment	Nu. myc.	Aer. myc.	Pigment
10.0	0.1	3	3	0	3	3	0	3	3–2 [2]	0
10.0	1.0	4	3	0	4	4	0	4	3	0
10.0	5.0	4	2	0	4	0	0	4	2	1 P
10.0	10.0	4	1	0	4	0	1 B	4	1	0
5.0	10.0	4	2	0	4	1–0	0	3	1	0
1.0	10.0	2	0	0	2	0	0	1	0	0
0.1	10.0	0	0	0	0	0	0	0	0	0

Grams per liter of medium		A-12 (saprophytic)			3374 (*A. praecox*) (saprophytic)			*A. setonii* (saprophytic)		
Dextrose	KNO_3	Nu. myc.	Aer. myc.	Pigment	Nu. myc.	Aer. myc.	Pigment	Nu. myc.	Aer. myc.	Pigment
10.0	0.1	3	2	0	3	2	0	3	3	0
10.0	1.0	5	5	2 P	5	5	0	5	5	0
10.0	5.0	5	5	2 P	5	3	0	5	5	0
10.0	10.0	5	5	1 P	5	3	0	5	5	0
5.0	10.0	5	5	0	5	2	0	5	5	0
1.0	10.0	3	3	0	3	2	0	3	3	0
0.1	10.0	1	1	0	1	0	0	1	0	0

[1] The amount of growth and intensity of pigmentation in Tables 13 and 14 are designated by the following numerals: 0. 1. 2. 3. 4. and 5. The color is indicated by letters: B—brown. P—pink
[2] Two numerals are used to show the variation in growth of cultures in the duplicate test tubes.

The results presented in Table 13, showing dextrose used with asparagine in seven different ratios, indicate that with ratios 10:0.1 and 10:1, the saprophytic and parasitic Actinomyces developed about the same amounts of nutritive and aerial mycelium. With a ratio of 10:5, the amount of growth in general was about the same as with the two previous ratios, except that the growth of aerial mycelium of parasitic cultures was considerably depressed. With ratios of 10:10 and 5:10, the parasitic cultures produced practically no aerial mycelium, but on the other hand the saprophytic cultures produced it to a maximum degree and especially at a 5:10 ratio. The amount of nutritive mycelium produced by the parasitic cultures was only slightly below that of saprophytic cultures.

The amount of nutritive mycelium decreased in both groups of organisms with a widening of ratios and when an excess of nitrogen was present. This was more pronounced for the parasitic cultures than for the saprophytes. The aerial mycelium of saprophytic cultures was developed in proportion to the amount of nutritive mycelium, while the parasitic cultures produced only traces of aerial mycelium. With ratios 10:5, 10:10, and 5:10, nutritive mycelium of parasitic cultures developed entirely on the surface and produced a much wrinkled layer. All the parasitic cultures produced a slight brown discoloration of the medium with a ratio of 10:5, and a stronger discoloration with a 10:10 ratio. Culture A-12 produced a pink discoloration with the first four ratios.

From this experiment, it is evident that with an abundant supply of carbon, the amount of nutritive mycelium of both groups of organisms gradually increased with an increase in the amount of nitrogen used. With a continued abundant supply of asparagine and a gradual decrease in dextrose, the amount of nutritive mycelium of the saprophytic cultures was slightly decreased, but noticeably so for the parasitic cultures. The saprophytic cultures developed aerial mycelium in abundance with practically all ratios (maximum with the ratio 5:10), except the 10:0.1 and 0.1:10 ratios. In contrast to the above, the aerial mycelium of the parasitic cultures reached its maximum growth with the ratio 10:1. It lessened progressively as the amount of nitrogen was increased and was practically absent in all ratios with a high amount of nitrogen. Table 14 presents the results of a similar test in which potassium nitrate was used instead of asparagine as the source of nitrogen. In the first four ratios of dextrose and potassium nitrate, with the same amounts of dextrose and with increasing amounts of potassium nitrate, the parasitic and saprophytic cultures showed the same tendencies toward development of nutritive and aerial mycelium as were shown with asparagine at the same ratios. However, with the ratio 10:10, two of the three parasitic cultures showed the presence of a small amount of aerial mycelium, which was entirely absent at the same ratio with asparagine.

With a ratio of 5:10 of dextrose and potassium nitrate, the growth of the saprophytic organisms was practically the same as it was with a ratio of 10:10, but the growth of nutritive mycelium of the parasitic cultures

was slightly decreased and the aerial mycelium slightly increased as with the 10:10 ratio. With a ratio of 1:10 there was a pronounced decrease in growth in both groups of organisms and both types of mycelium, and it was more pronounced for the parasitic cultures than for the saprophytic. The aerial mycelium of parasitic organisms was completely absent. With a ratio of 0.1:10 the decrease in growth in both groups of organisms was more pronounced than with a 1:10 ratio. The saprophytic organisms produced very poor growth with practically no aerial mycelium, and the parasitic cultures developed no growth at all. It would appear that the limiting factor of growth was an insufficient amount of available carbon (dextrose). With a similar ratio in which asparagine was used, the organisms showed more abundant growth than when potassium nitrate was used. It is possible that asparagine served as a source of nitrogen and carbon.

The nutritive mycelium of parasitic cultures with ratios 10:5, 10:10, and 5:10, did not produce the same peculiar wrinkled layer as when asparagine was used as a source of nitrogen. Culture 3374 (*A. praecox*) developed less aerial mycelium than the other two saprophytic cultures with all ratios. The following cultures produced various discolorations in the media at some of the ratios: culture 86 a slight brown color, and A-12 and 107 a slight pink color.

Conclusions.—In general, different ratios of carbon and nitrogen compounds had a pronounced effect on the development of nutritive mycelium, and from the data presented above the ratios of 10:1, 10:5, 10:10 and 5:10 appeared to be the most favorable for the development of nutritive mycelium for both groups of organisms.

There were no particular differences between parasitic and saprophytic cultures in their ability to produce nutritive mycelium with different ratios of carbon to nitrogen compounds. However, with very small amounts of carbon, parasitic cultures did not develop at all (see ratio 0.1:10 in Table 14).

The development of aerial mycelium of Actinomyces in general seemed to be dependent on certain ratios of carbon to nitrogen substances. It is also evident that a high amount of nitrogen had a suppressive effect on the development of aerial mycelium of parasitic cultures, and this effect was more pronounced when nitrogen was present in the form of asparagine, the organic source, rather than when potassium nitrate was used. At the same time a high nitrogen content did not have any retarding effect on the production of aerial mycelium of the saprophytic cultures. It is still not known whether or not this inhibiting effect of nitrogen is constant for all strains of *A. scabies* or only for those tested in this experiment. From numerous observations of the growth of different Actinomyces on media with a high nitrogen content, it appeared that this inhibiting effect represented a more general rule than an exception. It is therefore possible that the nitrogen content of a soil might have an important influence on the development of *A. scabies*.

PRODUCTION OF SOLUBLE PIGMENTS IN A TYROSINE MEDIUM

Certain organisms on media containing tyrosine produce a dark-brown to black pigment called melanin. This type of pigmentation was first observed by Krukenberg in 1878 (15) and he called it "melanose" or the process which leads to the production of melanin. Bertrand in 1896 (2) proved that this type of discoloration was due to the oxidation of tyrosine by the absorption of oxygen under the influence of a specific enzyme which he called "tyrosinase." In the case of Actinomyces, there are definite chromogenic cultures which are able to produce pigments on a tyrosine medium. According to Beijerinck (1) tyrosinase is a mixture of two oxidizing enzymes, one converting tyrosine into homogentisic acid and the other oxidizing the acid to melanin. Lutman and Cunningham (19) found that all strains of *A. scabies* were able to produce brown pigmentation on media containing peptones, proteins, and potato juice. Drechsler (8) found that saprophytic Actinomyces also exhibited a tyrosine reaction.

Waksman (33) found that when he used an alkali tyrosine agar (Krainsky, 1914) (14) only *A. scabies* and culture 205, out of 10 cultures used, produced a soluble brown pigment. The remaining eight cultures produced an abundant growth on this medium but did not produce a soluble pigment. He also pointed out that of four cultures of *A. scabies* which he obtained from different sources, only the two which grew the best gave the tyrosine reaction. He repeated this experiment using as a medium Czapek's Synthetic Solution, replacing sodium nitrate with 0.1 per cent of tyrosine and sucrose by 3 per cent of glycerol. He tested 10 different Actinomyces on this medium. One of three cultures of *A. scabies* produced a deep brown color, the other a pinkish color, and the third none. Only two or three other chromogenic cultures of Actinomyces were able to produce this pigmentation to any extent. He further states that not all of the cultures of Actinomyces which produced a brown to black soluble pigment on gelatin, potato plugs and on synthetic media containing organic substances, particularly proteins and peptones, were able to produce a soluble dark pigment on tyrosine.

Waksman assumes that only those Actinomyces which, according to Beijerinck, produced both enzymes, were able to transform tyrosine into melanin. He also states that all cultures of *A. scabies* isolated by him produced dark-brown pigments readily, but those obtained from other sources, especially when they were grown for several years on artificial media, seemed to lose this property entirely or to produce only a faint brown pigment. After reviewing Waksman's work on this subject, one must conclude either that only a few strains of *A. scabies* are able to produce melanin pigment on a tyrosine medium, or that when they are cultivated for a long period of time on an artificial medium they lose their ability to produce this pigment. Those strains of *A. scabies* which did not produce melanin pigment on a tyrosine medium were obtained by Waksman

from other sources and no statement was made regarding their pathogenicity.

In the present investigation a detailed study was made of the ability of parasitic and saprophytic Actinomyces to produce melanin pigment on a tyrosine medium, and of the possible existence of a correlation between the parasitism of Actinomyces and their ability to produce melanin pigment. The same basic medium as that described on page 44 was used with 15 grams of dextrose as a source of carbon. This medium was divided into two parts. To one part, designated as medium A, 1.0 gram per liter of tyrosine was added, and to the other, medium B, were added 1.0 gram of tyrosine and 3.0 grams of potassium nitrate. Medium B was used on the assumption that some of the cultures might not be able to use tyrosine as a single source of nitrogen, but probably could grow on a medium containing tyrosine in combination with potassium nitrate. The reaction of these two media was adjusted colorimetrically to pH 7.0 before sterilization. Two sets of test tubes, containing media A and B respectively, were inoculated with twenty-four different cultures of Actinomyces which had all been grown on the same medium for three weeks. Readings of the amount of nutritive and aerial mycelium produced, and the production of soluble pigments on the media were made periodically. The last readings on the growth of the organisms are presented in Table 15.

Practically all Actinomyces produced good growth on both media, but there was a noticeable difference in the production of soluble pigments. Only one culture, A-12, produced a slight yellow pigmentation in medium A (Table 15). When medium B was used, all seven of the parasitic cultures and one saprophytic culture, No. 90, produced a distinct dark brown to black pigment (melanin), and saprophytic cultures 88-A and 3369-A (*A. flavus*) produced a slight brown pigment. The intensity of pigmentation was greater with the parasitic than with the saprophytic cultures with the single exception of culture 90. In order to check the validity of these results, the experiment was repeated with the same medium but without agar. The cultures were grown for about four weeks. Table 15 gives the results of this experiment.

All the cultures which produced melanin pigment in the solid medium B produced the same discoloration in the liquid medium B with the exception that 3369-A (*A. flavus*) and some of the other cultures produced yellow, yellow-purple, and green pigments. Most of the cultures which produced melanin pigment in solid and liquid tyrosine media B, which contained potassium nitrate, produced only a slight yellow color in liquid medium A with only tyrosine as a source of nitrogen. It is possible that those cultures which produced a yellow pigment in liquid medium A also produced the same pigment in the similar solid medium, but it was not detected because the yellow color of the pigment was masked by the color of the agar itself. Practically identical results were obtained with solid and liquid media. The relative amount of growth of the different cultures was

TABLE 15.—*Growth of Actinomyces on tyrosine media.*[1]

Media	Parasitic cultures							Saprophytic cultures																		
	No. 2	79	86	87	104	105	107	89	90	103	106	3369 A *A. flavus*	*A. flavus* (Kr.)	*A. aeroni*	A-1	A-12	88-A	88-B	1356 *A. scabies*(?)	3369 B *A. flavus*	3372 *A. viridis*	3374 *A. praecox*	*A. claviger*	*A. tricolor*	*A. xantho-stroma*
Agar																										
Tyrosine (A)																										
Mycelium:																										
Nutritive	4	4	4	4	4	5	4	4	3	4	5	3	4	4	5	3	5	4	5	3	5	..	3	3	5
Aerial	1	2	4	2	2	4	4	2	4	3	4	0	2	3	4	3	4	0	1	0	5	..	0	4	4
Pigmentation	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1Y	0	0	0	0	0	..	0	0	0
Tyrosine + KNO_3 (B)																										
Mycelium:																										
Nutritive	4	5	4	4	4	5	5	4	5	5	5	4	2	5	5	5	2	1	3	2	4	..	4	2	4
Aerial	1	2	1	2	4	5	2	4	4	5	5	5	0	5	3	5	0	0	2	0	5	..	4	0	4
Pigmentation	5BBa	4BBa	5BBa	2BBa	4BBa	2BBa	4BBa	0	5BBa	0	0	1B	0	0	0	0	1B	0	0	0	4G	..	0	0	0
Solution																										
Tyrosine (A)																										
Mycelium:																										
Nutritive	5	5	4	4	5	5	5	3	4	4	3	3	3	5	5	3	4	4	3	3	5	5	3	3	4
Aerial	5	3	4	2	3	4	4	2	3	3	2	1	1	3	5	1	3	2	0	0	5	3	1	1	1
Pigmentation	2Y	1Y	1YB	2Y	1Y	1Y	1Y	0	0	0	0	1Y	1Y	0	1Y	0	0	0	0	0	0	0	0	0	0
Tyrosine + KNO_3 (B)																										
Mycelium																										
Nutritive	4	3	4	3	4	4	5	3	5	5	5	5	3	5	3	5	3	1	3	3	5	3	4	4	4
Aerial	4	1	0	1	2	3	3	4	4	4	5	3	1	5	2	5	1	0	1	0	4	0	3	2	2
Pigmentation	3DB	3DB	4DB	2DB	4DB	3B	4B	0	2B	1Y	4YPp	3Y	0	1Y	1YB	1Y	2DB	0	0	0	4G	0	0	0	0

[1] The amount of growth and intensity of pigmentation are designated by the following numerals: 0 none, 1 very slight, 2—slight, 3 fair, 4—good, and 5 very good. Pigmentation is indicated by the letters: Y—yellow, B—brown, Ba—black, G—Green, D—dark, Pp—purple.

approximately the same in the liquid as on the solid medium in both experiments. The intensity of melanin pigment in the solid medium was greater than in the liquid medium.

Effect of different amounts of potassium nitrate.—A new question arose when it was found that all parasitic cultures and three of the saprophytic cultures produced melanin discoloration only in a medium which contained both tyrosine and potassium nitrate. To what extent do each of these substances, tyrosine and potassium nitrate, influence the production of melanin pigment by Actinomyces, and why is melanin pigment not produced in a medium in which tyrosine is present alone?

In order to answer the first of these questions, a study was made to correlate the amount of melanin produced by cultures when different amounts of tyrosine and potassium nitrate were added to the agar of the same composition as was previously used. Four media containing the same

TABLE 16.—*Production of pigmentation and the growth of Actinomyces on media containing different amounts of tyrosine and* KNO_3.[1]

Media	Parasitic			Saprophytic			Parasitic			Saprophytic		
	N-23	86	107	A-12	3374 *A. praecox*	*A. setonii*	N-23	86	107	A-12	3374 *A. praecox*	*A. setonii*
1. Tyrosine 0.01 per cent, KNO_3 0.3 per cent							2. Tyrosine 0.05 per cent, KNO_3 0.3 per cent					
Mycelium:												
Nutritive	5	4	5	5	5	5	5	4	5	5	5	5
Aerial	3	0	3	5	5	5	3	0	3	5	4	5
Pigmentation	3B	4B	3B	2P	0	0	4BBa	5BBa	4BBa	3P	0	0
Final pH	9 4	9 4	9.4	9 4	9 4	9 4	9.4	9.4	9.4	9.4	9.4	9.4
NO_3	.	.	.	.	.	.	.	.	.			.
NO_2				. .	....		..	.				...
NH_3	.	.	.	.	.	.	.	.	.	.	.	.
3. Tyrosine 0.1 per cent, KNO_3 0.3 per cent							4. Tyrosine 0.2 per cent, KNO_3 0.3 per cent					
Mycelium:												
Nutritive	5	5	5	5	5	5	5	5	5	5	5	5
Aerial	3	1	2	5	4	5	3	0	3	5	4	5
Pigmentation	5BBa	5BBa	5BBa	3P	0	0	5BBa	5BBa	5BBa	4P	0	0
Final pH	9.4	9.4	9 4	9.4	9.4	9.4	9.4	9.4	9.4	9.2	9.4	9.2
NO_3						.						
NO_2	..				.							...
NH_3	.	.	.	.	.	.	.	.	.	.	.	
5. KNO_3 0.03 per cent, Tyrosine 0.1 per cent							6. KNO_3 0.15 per cent, Tyrosine 0.1 per cent					
Mycelium:												
Nutritive	5	5	5	5	4	5	5	4	5	5	5	5
Aerial	5	5	5	5	2	5	3	1	4	5	4	5
Pigmentation	2B	3B	2B	1P	0	0	4BBa	5BBa	3BBa	4P	0	0
Final pH	7 0	7 0	7 2	8 2	8 0	7 4	8 6	8 2	8 6	8.4	8 6	8 6
NO_3	0	0	0	0	0	0	.	.	.			
NO_2	0	0	0	0	0	0	.	.	0	.	.	.
NH_3	.	.	.	.	.	.	.	.	.	.	.	.
7. KNO_3 0.3 per cent, Tyrosine 0.1 per cent							8. KNO_3 0.6 per cent, Tyrosine 0.1 per cent					
Mycelium:												
Nutritive	5	5	5	5	5	5	5	5	5	5	5	5
Aerial	3	2	3	4	4	5	2	0	2	5	4	5
Pigmentation	5BBa	5BBa	5BBa	3P	0	0	5BBa	5BBa	5BBa	1P	0	0
Final pH	9 4	9 4	9 4	9 4	9 4	9 4	9 4	9 4	9 4	9 4	9 4	9 4
NO_3	.	.	.	.	.							
NO_2												
NH_3					.							

[1] The amount of growth and intensity of pigmentation are designated by the following numerals: 0—none, 1—very slight, 2—slight, 3—fair, 4—good, and 5—very good. Colors are indicated by the letters: B—brown, P—pink, Ba—black. The amount of nitrites, nitrates, and ammonia present in these media is designated as follows: 0—none, .—very slight, ..—slight, ...—good, ...—very good.

amounts of potassium nitrate combined with increasing amounts of tyrosine, and four other media with the same amounts of tyrosine combined with increasing amounts of potassium nitrate, were used (Table 16). Three parasitic cultures, N-23, 86, 107, and three saprophytic cultures, A-12, 3374 (*A. praecox*), and *A. setonii* were used in order to make a comparative study. The reaction of all eight media was adjusted to pH 7.0 before sterilization, and no changes in the reaction of the media occurred during sterilization. These cultures were grown for about one month in duplicate test tubes, and during this period three readings were made, the last of which are presented in Table 16.

The results of this experiment show that the growth in all 8 media was abundant. Both parasitic and saprophytic cultures produced a large amount of nutritive mycelium. The saprophytic cultures produced aerial mycelium to a maximum degree, while the parasitic cultures produced it only in varying amounts and always to a less degree than the saprophytes. With an increase in the amount of nitrogen, the amount of aerial mycelium of the parasitic cultures decreased. This tendency can be seen by examining sections 5, 6, 7, and 8 of Table 16, and the results are in accord with those obtained in the previous experiments, where the influence of different carbon-to-nitrogen ratios on the development of Actinomyces was studied.

Only parasitic cultures produced melanin pigment, and its intensity varied with the amounts of tyrosine and potassium nitrate present in the media. Sections 1 to 4 of Table 16 show that with a fixed amount of potassium nitrate and an increase in the amount of tyrosine, the intensity of melanin pigment increases until its maximum is reached with 0.1 per cent of tyrosine and 0.3 per cent of potassium nitrate (section 3). No further increase in the intensity of the melanin was observed with a higher concentration of tyrosine. Sections 5 to 8 show that with a fixed amount of tyrosine and with a gradual increase in the concentration of potassium nitrate, the intensity of melanin pigment increases until it reaches its maximum with the medium listed in section 7, which had the same amount of tyrosine and potassium nitrate as the medium listed in section 3. Culture A-12 produced a pink soluble pigment, the intensity of which was more or less correlated with the intensity of melanin produced by parasitic cultures. It is evident from the above that the amount of potassium nitrate, as well as that of tyrosine, influenced the production of melanin pigment.

At the end of this experiment the pH determinations of the media and qualitative determinations of the presence of nitrates, nitrites, and ammonia for all of these cultures were made, by the same methods as in the previous experiments. Traces of nitrates were present in all combinations with the exception of the medium which had only 0.03 per cent of potassium nitrate, in which nitrites were also absent. Nitrites were present to a slight degree with 0.15 per cent of potassium nitrate and very abundant in all the rest of the media where 0.3 per cent or more of potassium nitrate was added.

Culture A-12 showed an ability to use nitrites much more extensively than the other cultures, and this is in agreement with the results obtained in the previous experiments. A trace of ammonia was present in all media. The pH of the medium at the end of the experiment ranged from 7.0 to 8.2, when only 0.03 per cent of KNO_3 was added, and with increasing amounts the pH increased to a maximum of 9.4 with 0.3 per cent of KNO_3.

From these data it can be concluded that, first, practically all nitrates were reduced to nitrites; second, the range of pH can be correlated with the amount of potassium nitrate present in the medium (the less the potassium nitrate present, the lower the final pH of the medium); and third, a similar correlation can be shown to exist between the amount of potassium nitrate present and the production of melanin pigment by the parasitic cultures. The lower the pH and the smaller the amount of potassium nitrate, the less pigment produced.

The effect of potassium nitrate on the production of melanin pigment by Actinomyces in a tyrosine medium may be due either to (1) the oxidation of tyrosine by the oxygen liberated in the reduction of nitrates to nitrites or (2) to the increased alkalinity which favors the production of the melanin pigment.

Effect of other nitrogenous compounds.—In order to check the first of these two possibilities, another experiment was conducted in which the potassium nitrate (0.3 per cent) was replaced by a similar amount of the following nitrogenous compounds: calcium nitrate, sodium nitrate, ammonium sulphate, sodium nitrite, and ammonium carbonate (Table 17). The test was conducted as before and the same types of readings were taken. The last readings are presented in Table 17.

The organisms produced practically the same amounts of nutritive and aerial mycelium on media containing calcium nitrate, sodium nitrate, and ammonium carbonate. Only a fair amount of growth occurred with ammonium sulphate and none with sodium nitrite (Table 17). The absence of growth with sodium nitrite was probably due to the direct toxicity of nitrites to Actinomyces at this concentration, as reported by Waksman (33). The parasitic cultures, as usual, produced less aerial mycelium than the saprophytic, except on a medium containing calcium nitrate where the growth was about equal. The tendency of the parasitic cultures to develop more abundant aerial mycelium on media containing an excess of calcium was observed in other tests. The decrease in growth on a medium containing ammonium sulphate can be explained by the increase in acidity of the medium (pH 4.2) to a point which inhibits the development of most Actinomyces. The parasitic cultures started growth on the medium containing ammonium carbonate about one week later than the saprophytic cultures, but after growth had started it continued at about the same rate as the saprophytic cultures. Possibly either a direct toxic effect of the ammonia which forms in this medium, or an indirect effect of the same substance due to the sudden increase in alkalinity of

TABLE 17.—*Production of pigmentation and growth of Actinomyces on media containing tyrosine in combination with different nitrogenous compounds.*[1]

Media	Parasitic cultures			Saprophytic cultures			Check
	N-23	86	107	A-12	3374 *A. praecox*	*A. setonii*	
Tyrosine 0.1 per cent, $Ca(NO_3)_2$ 0.3 per cent							
Mycelium:							
Nutritive	5	4	5	5	5	5	—
Aerial	5	1	5	5	5	5	—
Pigmentation	3BBa	4BBa	2BBa	5PpCa	0	0	—
Final pH	9.2	9 2	9 2	9 2	9.2	9 2	6 0
NO_3	.	.	.	.	.	.	....
NO_2							.
NH_3	.	.	.	.	.	.	.
Tyrosine 0.1 per cent, $(NH_4)_2SO_4$ 0.3 per cent							
Mycelium							
Nutritive	4	3	3	4	3	3	—
Aerial	1	1	0	3	0	3	—
Pigmentation	1BBa	2BBa	1BBa	0	0	0	—
Final pH	4 2	4 2	4 2	4 2	4 2	4 2	6 8
NO_3	0	0	0	0	0	0	0
NO_2	0	0	0	0	0	0	0
NH_3				..	....	....	.. .

Media	Parasitic cultures			Saprophytic cultures			Check
	N-23	86	107	A-12	3374 *A. praecox*	*A. setonii*	
Tyrosine 0.1 per cent, $NaNO_3$ 0.3 per cent							
5	4	5	5	5	5	—	
2	1	2	5	4	5	—	
5BBa	5BBa	5BBa	2P	0	0	—	
9 4	9 4	9 4	9.4	9.4	9 4	7 0	
.	.	.	.	.	.	....	
....	....	....	...	....	....	.	
.	.	.	.	.	.	.	
Tyrosine 0.1 per cent, $(NH_4)_2CO_3$ 0.3 per cent							
5	4	4	5	5	5	—	
5	4	2	5	5	5	—	
5BBa	5BBa	5BBa	2YB	3YB	2YB	—	
9 2	9 2	9 2	9 2	9 2	9 2	7 7	
0	0	0	0	0	0	0	
0	0	0	0	0	0	0	
....	...	....	.	..	....	....	

[1] No growth occurred on a medium containing 0.3 per cent sodium nitrite. The amount of growth and intensity of pigmentation are designated by the following numerals: 0—none, 1—very slight, 2—slight, 3—fair, 4—good, and 5—very good. Pigments are indicated by B—brown, Ba—black, Pp—purple, Ca—carmine, P—pink, Y—yellow. The amount of nitrates, nitrites and ammonia present in these media is designated as follows: 0—none, .—very slight, —slight, good, ...—very good.

the medium, was responsible for this irregularity in the growth of parasitic cultures.

The intensity of the melanin pigment which the parasitic cultures developed varied considerably with different substances used. They produced a maximum discoloration with sodium nitrate and ammonium carbonate, less with calcium nitrate, and only a trace with ammonium sulphate. Thus it is evident that parasitic Actinomyces can transform tyrosine into melanin in a medium where tyrosine is present in combination with nitrogenous salts other than nitrates. This indicates that the reduction of nitrates to nitrites, and the available oxygen produced in this process, are not necessary in the formation of melanin from tyrosine. A higher alkalinity was always associated with a greater production of melanin by parasitic cultures. In a medium containing ammonium sulphate, growth and discoloration were present only on the surface of the agar. This was probably due to a small amount of ammonia undoubtedly present in this part of the medium, which made the reaction at the surface slightly alkaline or not quite as acid as in the lower part of the medium.

The final pH reading showed a high degree of alkalinity in media containing calcium nitrate, sodium nitrate, and ammonium carbonate and a strong acid reaction in a medium to which ammonium sulphate was added. The reaction of the medium containing sodium nitrite was unchanged. Media containing nitrates at the beginning of the experiment had practically all of their nitrates reduced to nitrites. Culture A-12, as in previous experiments, showed a greater ability to use nitrites than any of the other cultures. Media containing different ammonium compounds showed an excess of ammonia at the end of the experiment.

Because of the apparent toxicity of nitrites at the concentration used in the previous experiment, another test was made in which the 0.3 per cent sodium nitrite was replaced with 0.005 per cent, which has been reported by Waksman (33) to be nontoxic. The same six cultures and the same procedure were used. All Actinomyces tested grew abundantly on this medium and all parasitic cultures produced a slight melanin discoloration (Table 18). From this experiment it is evident that nitrites at a concentration of 0.005 per cent are not toxic to Actinomyces, and that parasitic cultures are able to produce melanin pigment on tyrosine medium in combination with nitrites.

The results of these experiments show that the extent of melanin pigment production is correlated with the ability of different nitrogenous compounds to develop an alkaline reaction of the medium. Further tests of the relation of alkalinity to pigment production in a tyrosine medium without any other source of nitrogen were therefore conducted.

Effect of alkalinity.—The three tyrosine media that were used in the previous experiments were tested without any other sources of nitrogen. The reactions of these media were adjusted after sterilization with dilute

TABLE 18.—*Production of pigmentation and the growth of Actinomyces on a medium containing tyrosine in combination with sodium nitrite.*[1]

Media	Parasitic cultures			Saprophytic cultures			Check
	N-23	86	107	A-12	3374 *A. praecox*	*A. setonii*	
Tyrosine 0.1 per cent, $NaNO_2$ 0.005 per cent.							
Mycelium:							
Nutritive	5	5	5	5	5	5	—
Aerial	2	4	2	5	1	5	—
Pigmentation	1B	3B	1B	0	0	0	—
Final pH.....	6.8	7.4	6.8	7.0	6.2	6 0	7.2
NO_3	0	0	0	0	0	0	0
NO_2	0	0	0	0	0	0	....
NH_3	.	.	.	.	.	.	.

[1] The amount of growth and intensity of pigmentation are designated by the following numerals: 0—none, 1—very slight, 2—slight, 3—fair, 4—good, and 5—very good. The amount of nitrates, nitrites, and ammonia present in this medium was designated as follows: 0—none, .—very slight, ..—slight, ...—good, ...—very good. Color is indicated by B—brown.

sodium hydroxide to pH 7.0, 8.0, and 9.0. The same six cultures and the same procedure as in the previous tests were used. Final readings of this experiment are given in Table 19.

Pigmentation, except that produced by culture 86, was very slight and developed only in a medium with pH 8.0 and 9.0. This indicates that the high alkalinity alone is not sufficient to insure the extensive production of melanin pigment by parasitic cultures in a tyrosine medium.

In conclusion it is possible to say that the role of nitrogenous compounds in the development of melanin pigment by parasitic cultures on a tyrosine medium is probably helpful in changing the reaction of a medium

TABLE 19.—*Production of pigmentation and the growth of Actinomyces on tyrosine media with different pHs.*[1]

pH	Parasitic cultures			Saprophytic cultures			Check
	N-23	86	107	A-12	3374 *A. praecox*	*A. setonii*	
pH 7.0							
Mycelium:							
Nutritive	5	4	5	5	5	4	—
Aerial	2	2	1	2	2	4	—
Pigmentation	0	1YB	0	0	0	0	—
Final pH.....	6.4	6.6	6.2	6.6	6.0	6 0	6 6
pH 8.0							
Mycelium:							
Nutritive	5	5	5	4	5	5	—
Aerial	2	3	3	4	2	5	—
Pigmentation	0	3B	0	0	0	0	—
Final pH	7.0	7.4	7.0	7.4	6 0	6.4	7.6
pH 9.0							
Mycelium:							
Nutritive	5	4	5	4	5	5	—
Aerial	4	4	4	4	2	5	—
Pigmentation	1B	3B	1B	0	0	0	—
Final pH	7.8	7.9	7.7	7.5	6.8	7.1	7.8

[1] The amount of growth and intensity of pigmentation are designated by the following numerals: 0—none, 1—very slight, 2—slight, 3—fair, 4—good, and 5—very good. Colors are indicated by B—brown, Y—yellow.

to the alkaline side and in this way producing favorable conditions for the devolopment of melanin pigment. At the same time it is possible that these nitrogenous compounds provide a readily available source of nitrogen which protects tyrosine from being used as a source of nitrogen for the growth of the organism until the reaction of the medium reaches a favorable point for melanin formation.

Effect of different carbon compounds.—In all of these experiments with tyrosine, dextrose was used as the only source of carbon. It was, however, not known whether different sources of carbon have any importance in the production of melanin pigment on a tyrosine medium. In order to investigate this point, the following experiment was conducted. The usual tyrosine-potassium-nitrate medium was used, but instead of dextrose the same amounts of the following sources of carbon were substituted: arabinose, galactose, sucrose, maltose, starch, and xylan. These carbohydrates were sterilized with dry heat at 105° C. and added to the six previously sterilized media. The same six cultures were used and the experiment was conducted in the same manner as the previous tests. Table 20 presents the results of this test.

Practically all organisms, with the exception of the saprophytic cultures on the sucrose medium, developed a good growth. In contrast to the previous experiments, saprophytic cultures were able to grow on the sucrose medium, probably because they were able to use tyrosine as a source of carbon. There were no striking differences in the production of melanin pigment on these media. The slight variation in the production of melanin was probably due to the fact that there is a difference in the ability of parasitic Actinomyces to utilize various carbohydrates as a source of carbon, so that it would naturally be expected that a variation in the metabolic processes would occur. The final reaction of all cultures grown on all media was pH 9.2, except for the saprophytic cultures which were grown on a medium containing sucrose and xylan, and where the reaction was less alkaline. Nitrates were used more extensively by the parasitic Actinomyces than by the saprophytic cultures, but nitrites apparently were used more abundantly by the saprophytes.

TABLE 20.—*Production of pigmentation and the growth of Actinomyces on media containing tyrosine and KNO_3 in combination with different sources of carbon.*[1]

Source of carbon	Parasitic cultures			Saprophytic cultures			Check	Parasitic cultures			Saprophytic cultures			Check
	N-23	86	107	A-12	3374 *I. praecox*	*A. setonii*		N-23	86	107	A-12	3374 *A. praecox*	*A. setonii*	
Arabinose								**Galactose**						
Mycelium:														
Nutritive	5	4	5	5	5	5	—	5	5	5	5	5	5	—
Aerial	2	0	2	5	4	5	—	2	1	3	5	5	5	—
Pigmentation	5BBa	5BBa	4BBa	1PY	0	0	—	3BBa	5BBa	3BBa	2PY	0	0	—
Final pH	9.2	9.2	9.2	9.2	9.2	9.2	7.0	9.2	9.2	9.2	9.2	9.2	9.2	7.0
NO_3	.	.	.	.	.	.	....	.	.	.	.	..	.	....
NO_2	.	.	.	.	.	.	0	....	....	....	.	...	...	0
NH_3	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Sucrose								**Maltose**						
Mycelium:														
Nutritive	5	4	5	1	2	3	—	5	5	5	5	5	5	—
Aerial	1	0	2	0	0	3	—	1	4	5	5	5	5	—
Pigmentation	5BBa	5BBa	5BBa	0	0	0	—	4BBa	4BBa	3BBa	3PY	0	0	—
Final pH	9.2	9.2	9.2	8.0	8.0	8.0	7.2	9.2	9.2	9.2	9.2	9.2	9.2	7.0
NO_3	.	.	.	.	.	.	....	0	0	0	0	..	.	....
NO_2	.	.	.	.	..	..	0	....	....	....	.	...	...	0
NH_3	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Starch								**Xylan**						
Mycelium:														
Nutritive	5	5	5	5	5	5	—	4	4	4	4	4	4	—
Aerial	2	2	3	5	5	5	—	2	2	2	4	3	4	—
Pigmentation	5BBa	5BBa	5BBa	2PY	0	0	—	4BBa	4BBa	4BBa	0	0	0	—
Final pH	9.2	9.2	9.2	9.2	9.2	9.2	7.0	9.2	9.2	9.2	8.4	8.4	8.4	7.0
NO_3	.	.	.	.	.	.	....	.	.	.	....	....	....	....
NO_2	.	.	.	.	.	.	0	....	....	....	...	.	..	0
NH_3	.	.	.	.	.	.	.	.	.	.	.	.	.	.

[1] The amount of growth and intensity of pigmentation are designated by the following numerals: 0—none, 1 very slight, 2—slight, 3—fair, 4—good, and 5—very good. Colors are indicated by B—brown, Ba—black, P—pink, and Y—yellow. The amount of nitrates, nitrites, and ammonia present in these media is designated as follows: 0—none, .—very slight, ..—slight; ...good, and ...—very good.

PRODUCTION OF SOLUBLE PIGMENTS WITH AMINO ACIDS OTHER THAN TYROSINE

In connection with the previous experiments the question arose as to whether there are amino acids other than tyrosine that are favorable for the production of similar pigments by the action of Actinomyces. In order to answer this question the following experiment was conducted.

The same basic medium was used with the following amino acids instead of tyrosine: phenylalanine, histidine-hydrochloride, and tryptophane. Duplicate sets of these different media were used, with and without potassium nitrate. The same twenty-five cultures of Actinomyces were tested. The results are presented in Table 21, and show that when amino acids without potassium nitrate were used, only a few cultures developed slight yellow or slight brown pigmentations in the media, but when they were used in combination with potassium nitrate, pronounced brown or yellow pigmentations were developed by practically all parasitic and many saprophytic cultures. Pigmentation was slight in media containing phenylalanine with potassium nitrate or histidine-hydrochloride with potassium nitrate, but was abundant in a medium containing tryptophane with potassium nitrate. Practically all the parasitic and a number of the saprophytic cultures developed a distinct, brown pigment on a trytophane medium with potassium nitrate.

In general, it is possible to say that Actinomyces grown on media containing the above-mentioned amino acids alone or in combination with potassium nitrate, failed to develop pigments comparable to melanin.

CONCLUSIONS RELATING TO PIGMENT FORMATION

The cultures of Actinomyces used in this investigation failed to develop melanin pigment in a medium containing tyrosine alone, but this pigment was produced in abundance when tyrosine was present in combination with other nitrogenous compounds. The nitrogenous compounds probably were helpful in changing the reaction of a medium toward the alkaline side and in this way produced conditions favorable for the development of melanin pigment. At the same time it is possible that by furnishing an easily available source of nitrogen, the tyrosine was not used until the reaction of the medium reached a point favorable for melanin production.

The various sources of carbon had only an indirect influence on the development of melanin pigment, according to their fitness as a source of carbon for Actinomyces. Actinomyces cultures tested on amino acids other than tyrosine, failed to develop pigments comparable to melanin.

In this study all of the parasitic cultures and three of the 18 saprophytic cultures produced melanin pigment in a tyrosine medium. If all parasitic cultures produce melanin pigment and only a few saprophytic cultures do so, this would be of value in differentiating parasitic from saprophytic Actinomyces. As far as the author is aware, Millard and

TABLE 21.—*Production of pigmentation and the growth of Actinomyces on media containing different amino acids as such and in combination with KNO_3.*[1]

Media	Parasitic cultures							Saprophytic cultures																
	N-23	79	86	87	104	105	107	89	90	103	106	3369-A *A. flavus*	*A. flavus* (Kr.)	*A. setonii*	A-1	A-12	88-A	88-B	1356 *A. scabies*(?)	3369-B *A. flavus*	3372 *A. viridis*	3374 *A. praecox*	*A. claviger*	*A. tricolor*	*A. xantho-stroma*
Phenylalanine without KNO_3																									
Mycelium:																									
Nutritive	5	3	5	4	5	4	4	1	4	4	4	4	5	4	5	4	1	1	4	4	5	4	3	5	5
Aerial	5	3	5	4	4	4	4	0	4	4	4	0	4	2	5	0	0	0	3	0	5	0	0	5	5
Pigmentation	0	0	0	0	0	1Y	0	0	0	0	0	1Y	1Y	0	0	1C	0	0	1Y	0	1G	0	0	1Bl	0
Phenylalanine with KNO_3																									
Mycelium:																									
Nutritive	5	5	5	1	5	5	4	5	4	5	5	5	5	5	5	5	1	1	5	4	5	4	5	1	4
Aerial	3	2	4	0	5	5	4	5	5	5	5	5	3	5	5	5	0	0	4	0	5	4	1	0	4
Pigmentation	2B	2B	3B	0	1B	2B	1B	1B	2B	2B	2B	2Y	1Y	1B	1B	1C	0	0	0	0	2G	2B	1B	0	0
Histidine hydrochloride without KNO_3																									
Mycelium:																									
Nutritive	5	4	4	4	5	5	4	3	4	5	4	4	5	5	5	5	4	5	5	4	5	4	4	5	5
Aerial	5	3	4	3	4	5	4	0	4	4	5	3	3	5	5	5	3	4	4	0	5	2	4	4	5
Pigmentation	0	0	0	0	0	0	0	0	0	0	0	1Y	1Y	0	0	1C	0	0	2Y	0	2G	0	0	2Bl	0
Histidine hydrochloride with KNO_3																									
Mycelium:																									
Nutritive	3	3	4	3	4	5	4	5	4	5	5	5	5	5	5	5	1	1	4	4	5	5	5	1	5
Aerial	1	2	2	2	3	4	5	5	4	5	5	5	2	5	5	5	0	0	4	0	5	5	5	0	5
Pigmentation	1B	1B	2B	0	2B	2B	2B	0	1B	1B	2B	1B	0	0	1Y	2C	0	0	1B	1Y	3G	0	1Y	0	0
Tryptophane without KNO_3																									
Mycelium:																									
Nutritive	5	4	4	3	4	4	5	3	2	4	2	3	4	2	5	3	2	1	3	3	4	2	5	4	3
Aerial	4	2	5	3	4	3	4	0	0	0	0	1	1	2	5	1	0	0	0	0	4	1	5	3	1
Pigmentation	0	0	0	0	0	1Y	0	0	0	0	0	0	1Y	0	0	0	0	0	1B	0	2G	0	0	0	0
Tryptophane with KNO_3																									
Mycelium:																									
Nutritive	5	4	5	4	5	5	5	5	5	5	5	4	5	5	4	5	2	2	3	3	5	5	4	2	3
Aerial	2	2	2	2	3	3	2	5	3	5	5	5	2	5	1	2	0	0	0	0	5	3	1	0	1
Pigmentation	5B	4B	5B	0	4B	4B	4B	0	3B	0	0	3B	0	0	1B	3B	0	0	0	0	1G	0	1B	0	0

[1] The amount of growth and intensity of pigmentation are designated by the following numerals: 0—none, 1—very slight, 2—slight, 3—fair, 4—good, and 5—very good. Colors are indicated by Y—yellow, C—cream, G—green, Bl—blue, B—brown.

Burr's investigation (22) is the only one which indicates that nonchromogenic cultures of Actinomyces are also parasites, but the author's investigation failed to prove the pathogenicity of their cultures which were used in this work.

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